THE IMMUNOBIOLOGY OF A SQUAMOUS CELL CARCINOMA

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DOCTOR OF PHILOSOPHY

by

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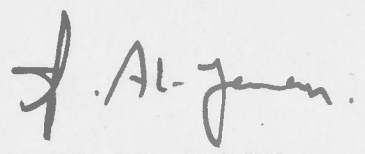
STATEMENT

The ultrastructural studies of the cultivated tumour cells were done jointly with Professor Bede Morris. Miss W. Trevella assisted with the cannulations of the lymphatic vessels in sheep. The histological sections were prepared by Miss W. Hughes. All the other work reported in this thesis was done by myself.



FADWA AL-YAMAN

TO MY FATHER.

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ABSTRACT

This thesis reports experiments I have done to investigate some of the immunobiological aspects of a naturally occurring squamous cell carcinoma in sheep. Methods were established for the successful cultivation of a number of pure populations of tumour cells *in vitro*. The epithelial nature of the established cell lines was confirmed by electron microscopic studies. An association was found between the site at which the tumour grew on the host and the rate of success obtained in establishing the tumour in culture. Tumours growing on the muzzle were established in culture most easily followed by those on the ear and the vulva respectively. A similar correlation was observed between the site of the primary tumour and its capacity to grow in nude mice.

Studies on the tumour cell-lymphocyte interaction indicated that all tumour cell lines that were tested failed to stimulate a proliferative response in allogeneic lymphocytes when mixed together *in vitro*. This lack of stimulation was found to be due to an immunosuppressive capacity of the majority of the cell lines tested. This immunosuppressive capacity of the tumours, which was manifest *in vitro*, was not seen *in vivo*, since challenge of allogeneic normal sheep with tumour cells resulted in significant stimulation of the recipient's immune system.

The immune reactivity of tumour-bearing sheep to autochthonous tumour cells was investigated both in the lymph node regional to the tumour and in lymph nodes distant from the tumour site. This was done by cannulating both the afferent and the efferent lymphatics of the lymph nodes and measuring the immune responses

that were produced, in the efferent lymph following challenge with autochthonous tumour cells. While the primary tumour remained present, no immune reactivity, as measured by cellular changes in the lymph from the regional nodes was detected whereas a significant response did occur in the lymph from a distant node when this was challenged directly with tumour cells. The response observed in the distant lymph node was associated with the production of antibodies which bound to the tumour cells but were not cytotoxic for them. Removal of the primary tumour and subsequent sensitization of the host three weeks after the tumour was resected, resulted in the appearance of specific humoral and cellular cytotoxic mechanisms in the efferent lymph from distant nodes when these were challenged by autochthonous tumour cells.

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CHAPTER 1

INTRODUCTION

Skin cancer is one of the most common cancers of man. The incidence of skin cancer in Australia is the highest of any country in the world and represents more than 50% of all the cancers diagnosed. There is a well established association between the incidence of skin cancer in man, and the degree to which the skin is exposed to sunlight. Skin cancer in sheep is also common in Australia and this tumour, like the skin tumours in man, has been attributed to the effect of ultraviolet irradiation of the sun. Thus skin cancer in sheep provides an excellent model for studying the biology of a cancer which is of important medical significance (Ladd and Entwistle 1977).

The aim of the work described in this thesis was to investigate some of the immunobiological aspects of this naturally occurring solid tumour of the skin of sheep. The introduction to the thesis consists of two parts; one part describes the histological structure of the skin, the alterations in the skin which are a prelude to the development of either basal or squamous cell carcinomata, and includes a discussion of the natural history of squamous cell carcinoma in sheep. The other part reviews the various types of immune reactivities detected in natural and experimental tumours both *in vitro* and *in vivo*.

1.1 PATHOLOGY OF SKIN TUMOURS

The skin consists of an outer epidermis and an inner dermis. The epidermis is divided into four main layers; the basal layer consisting of a single row of columnar epithelial cells (stratum germinativum), a thick prickle cell layer (stratum spinosum), a thin layer of diamond-shaped cells filled with granules known as

keratohyalin (stratum granulosum) and, a surface layer of dead keratinized cells (stratum corneum). Cell proliferation in the epidermis occurs mainly in the basal layer. Newly-formed cells leave this layer and move outwards through the more superficial layers until they reach the surface and are shed. During this process of outward migration, the cells undergo a series of characteristic morphological changes in which they become progressively more keratinized. As the cells produce the insoluble protein keratin, they lose their nuclei and die. Thus, under normal conditions, the production of new cells in the basal layer is balanced against the death of cells through the process of keratinization and desquamation. In certain circumstances, for example following prolonged exposure of the skin to UV-light, hyperplasia of the epidermis can occur (Blum 1959; Blum *et al.*, 1975). This hyperplastic condition of the skin may, with time, revert to normal or become permanent. Two types of epidermal carcinomata may result from alterations in the balance between cell proliferation and cell differentiation (keratinization), and these tumours are classified as basal cell and squamous cell carcinomata.

1.1.1 Basal Cell Carcinoma

Basal cell carcinoma accounts for over 75% of all skin cancers (Fitzpatrick and Pathak 1977). These carcinomata arise from the basal layer of the epidermis and the malignant cells show little tendency to undergo the usual differentiation into keratinized squamous cells (Belisario 1959; Klein *et al.*, 1973). Although these tumours very rarely metastasise, they are locally invasive and, if neglected, they will spread into underlying

structures including nerves, bone and brain. This cancer occurs mainly on the head and neck of man, classically distributed around the shade line of the hat. The typical basal cell carcinoma appears as a non-inflamed smooth, translucent waxy nodule, which has a variable amount of melanin pigment. Such nodules often ulcerate and a crust forms on their surface. Basal cell carcinomata may also take other forms, including more subtle infiltrating lesions that do not produce elevated nodules. Although there is no readily recognizable premalignant lesion that precedes a basal cell carcinoma, the cancer is normally seen in people who manifest symptoms of skin damage from sunlight or X-rays (Klein *et al.*, 1973).

1.1.2 *Squamous Cell Carcinoma*

Squamous cell carcinoma also arises from the epidermis and the malignant cells show significant differentiation into squamous cells. This cancer is characterized histologically by the presence of accumulations of keratinized cells with a characteristically whorled arrangement described as keratin pearls (Klein *et al.*, 1973; Fitzpatrick and Pathak, 1977). This pattern is apparent in the early stages of the disease. Squamous cell carcinoma may also grow and infiltrate the deeper layers of the dermis and subcuticulum as does the basal cell carcinoma or it may grow to form a raised hyperplastic lesion which may infiltrate the deeper tissue at a later stage. Squamous cell tumours tend to metastasise more readily than basal cell tumours, but the outcome depends on the size, location and the invasiveness of the tumour. In contrast to basal cell carcinomata, squamous cell carcinomata most

commonly arise from a pre-existing actinic or solar keratosis. These premalignant keratoses are scaly, rough, red plaques that occur in chronically sun-damaged skin. Although very few of these keratoses progress to carcinomata, most squamous-cell carcinomas on exposed skin do arise from these keratoses (Klein *et al.*, 1973; Scott and Starf 1977). The type of squamata which arise from keratotic lesions has a low frequency of metastases (below 2%, Fitzpatrick and Pathak 1977). Squamous cell carcinomata which arise from mucous membranes, mucocutaneous junctions, burn scars or chronic ulcers have a greater tendency to metastasize (Klein *et al.*, 1973). In general however, the malignancy of squamous cell carcinomata is fairly low and metastases occur infrequently. When the tumours do spread, they do so principally through the lymphatic system although occasionally they do spread by way of the blood. Because of this metastases are seen most frequently in the lymph nodes regional to the tumour but they occur sometimes in the lungs. Secondary growths are rarely found outside these two sites.

1.2 THE AETIOLOGY OF SKIN CANCER IN MAN

1.2.1 Ultraviolet Irradiation

It has been suspected for many years that the ultraviolet component of sunlight has carcinogenic activity (Blum 1948; 1964). Evidence to support this proposition is derived from both epidemiological and experimental studies. Epidemiological observations have shown that there is a close correlation between the extent of exposure of skin to sunlight and the incidence of skin cancer. Studies have shown that the incidence of skin cancer

in outdoor workers is much higher than in people who work indoors (Upton 1973; Urbach 1975) and that the lesions occur most commonly on those parts of the body which are most exposed to sunlight. The areas most commonly affected are the skin of the face, particularly the ear, lip and nose, the backs of the hands and the back of the neck (Silverstone and Gordon 1966; Urbach 1975). The relationship between the exposure to sunlight and the prevalence of skin cancer is also highlighted by the high incidence of these tumours in persons living in the tropics (Upton 1973; Scott and Starf 1977). The fact that blonde races with little skin pigmentation are more susceptible to skin cancer than dark-skinned people also suggests that UV-light is a causative agent of the disease. The highly susceptible people are those who have fair complexions and who sunburn easily, severely and often (Silverstone *et al.*, 1963; Silverstone and Searle 1970). Furthermore, people suffering from xeroderma pigmentosum (XP), an inherited disease characterized by extreme sensitivity of the skin to sunlight, show a significantly higher incidence of skin cancer than the normal population (Robbins *et al.*, 1974; Burnet 1978; Setlow 1978). Skin cancer has been produced experimentally in laboratory animals by exposing them to UV-irradiation and this finding supports the causal relationship between UV-light and skin cancer (Blum 1959; Kripke 1974).

1.2.2 *Photosensitization*

Photosensitization is another factor which has been implicated in the causation of skin cancer (Weisburger 1973). Cutaneous photosensitivity is a general term used in referring to the abnormal reaction of the skin to light. The adverse response

manifested by the skin is usually the result of the exposure to certain chemical agents coupled with sunlight. This can occur if the photosensitizing agents are present either in the interstitial fluid of the skin or in the epidermal cells themselves. Thus the sensitizing agent can arrive in the skin following absorption into the blood or following topical application. Photosensitivity reactions are characterized by an abnormal sunburn reaction, oedema of the skin and an acute eczematous reaction. This may be accompanied by desquamation and the subsequent hyperpigmentation of healed lesions.

Many substances which occur in nature (plants and certain oils) have a photosensitizing potential. Many other substances which have been prepared synthetically for clinical and commercial purposes can also cause photosensitivity reactions when the skin is exposed to sunlight. These include certain chemical agents such as tranquillizers, antibiotics and antimicrobial agents that are incorporated into soaps and other cosmetic products. These agents or drugs are generally innocuous to the skin in the absence of exposure to light. However, if the skin is subjected to a sufficient concentration of the agent together with the appropriate wavelength of light, photosensitivity reactions occur and the cells may be damaged or killed.

There are experimental studies as well as clinical observations which suggest that photosensitization may play a role in carcinogenesis. Photosensitizing substances result in skin damage and damaged skin seems to predispose to skin cancer (Klein *et al.*, 1973; Weisburger 1973). Thus most squamous cell carcinomata arise from skin that has been damaged by sun or from pre-cancerous lesions; they rarely appear in areas which are completely

normal (Willis 1948; Belisario 1959; Klein *et al.*, 1973). Experimentally, Büngeler (1937) observed an increased incidence of epithelioma in the skin of mice treated with chemical carcinogens following photosensitization with eosin and haematoporphyrin. Thus it seems that contact with photosensitizing substances may provoke lesions of the skin; such lesions may become cancerous when exposed to other carcinogenic agents. Consequently, photosensitizing substances may play a role in cancer development by acting as co-carcinogens.

1.3 OVINE SQUAMOUS CELL CARCINOMA

1.3.1 Introduction

Epithelial skin tumours are common neoplasms affecting domestic animals and have been described in a variety of animal species such as cattle (Drabble 1929; Anderson *et al.*, 1956; French 1959), dogs (Nielsen and Cole 1960), goats (Ramadan 1975), horses (Feldman 1926) and sheep (Dodd 1923; Davis and Shorten 1952; Carter 1958; Lloyd 1961; Vandegraaff 1976; Ladd and Entwistle 1977). In all these species, as in man, the tumours are common on those parts of the body which lack protective pigmentation and are exposed to the greatest intensity of sunlight (Dodd 1923; French 1959; Lloyd 1961; Ladd and Entwistle 1977). This epidemiological finding supports the view that solar irradiation is a causative factor in skin cancer in animals as well as in man.

The details of squamous cell carcinomata in sheep have been reported by several investigators (Dodd 1923; Carter 1958; Lloyd 1961; Vandegraaff 1976; Ladd and Entwistle 1977).

Extensive studies on this tumour were done by Lloyd (1961) and by Ladd and Entwistle (1977). Lloyd (1961) studied a flock of 7840 Merino sheep in which an increase in the incidence of the tumours was observed following a period of drought conditions. The overall incidence of the tumours in the flock was 1.75% compared with the usual incidence of approximately 0.2%. Ladd and Entwistle (1977) studied the disease over a period of 4 years in a flock of 8000 Merino sheep maintained at a research station in Queensland.

In the studies of both Lloyd (1961) and Ladd and Entwistle (1977) a number of parameters associated with malignancy were investigated. These are discussed below.

1.3.2 *Age and Sex Incidence of Ovine Squamous Cell Carcinoma*

Both studies showed an increased incidence of tumours in older sheep. The maximum age of sheep in the flock studied by Lloyd (1961) was 6 years. In this age group, the incidence of the tumour was 3.26%. No tumours were observed in sheep between 1-3 years old. The age of the sheep investigated by Ladd and Entwistle extended up to 12 years. In the 12 year old sheep, the incidence of tumours was as high as 12%. The increased incidence of ovine squamous cell carcinoma in the oldest age group studied was significant and this finding was similar to the increased incidence of tumours with age in man (Silverstone *et al.*, 1963; Silverstone and Searle 1970; Möller and Möller 1975).

Ladd and Entwistle (1977) suggested that ewes were more susceptible to the disease than wethers. However, the unequal distribution of sexes within the flock studied could have influenced the interpretation of results obtained.

1.3.3 Site of Lesions

A total of 69 and 146 tumours were observed by Lloyd (1961) and Ladd and Entwistle (1977) respectively. In both studies it was found that the most common site for tumours was the ear, followed by the muzzle, the lower lip, perineal region and finally, the eye. When tumours were present on the ears, they were mainly located on the outer aspect of the auricle and on the distal part (Lloyd, 1961). The sites of the lesions were consistent with the idea that sunlight is an important factor in the aetiology of the disease, since the outer aspect of the auricle and the distal part of the ear would be more exposed to sunlight than the inner aspect of the auricle and the proximal part. Lloyd (1961) has pointed out that the lower incidence of the tumours on the muzzle as compared to the ears may be due to the fact that on hot days sheep move around with their heads down, and this reduces the extent to which the skin of the muzzle is exposed to sunlight. Moreover, the wool on the fronto-parietal region and the wool covering the cheeks seems to provide protection for the eye from sunlight and this may explain the low incidence of tumours in that region (Lloyd 1961).

The perineal region of sheep is not usually exposed to much direct sunlight and the incidence of tumours in this region is low. It is a management practice in some flocks to perform the Mule's operation to remove skin wrinkles from each side of the vulva and across the tail so as to produce a woolless perineum which is stretched free of skin folds. This operation is performed to reduce the incidence of blow-fly strike. The removal of medial perineal skin folds during the Mule's operation leads to lateral contraction of the skin and partial opening of the lips of the vulva during the healing process. This results

in an increased exposure of this region to sunlight and as a result up to 3% of 3 year old ewes may have tumours of the vulval region (Vandegraaff 1976).

1.3.4 Frequency of Metastases

The frequency of metastases of skin tumours in sheep is approximately 11-12% (Lloyd 1961; Ladd and Entwistle 1977). The secondary tumours occur mainly in the regional lymph nodes particularly in the parotid, retropharyngeal, mandibular and prescapular lymph nodes. A single case was described in which the secondary lesion was found in the lung (Lloyd 1961).

Lloyd (1961) suggested that the actual frequency of metastases in sheep might be higher than 12% since two factors would reduce the number of secondary tumours observed, the early death of the affected animals due to infection or fly strike, and early removal of lesions by the stockowner. These two factors make it difficult to obtain an accurate figure for the incidence of secondary tumours in sheep with skin cancer.

1.3.5 Aetiology

There are probably several factors which are responsible for the aetiology of ovine squamous cell carcinoma; however as in man, squamous cell carcinoma in sheep has been mainly attributed to the effect of solar UV-irradiation (Dodd 1923; Lloyd 1961; Ladd and Entwistle 1977) although other contributing factors have been implicated.

Carne *et al.* (1963) suggested that penetration

of the skin by grass seeds was closely associated with the development of skin cancer in sheep. Dodd (1923), examined 47 skin tumours in sheep and observed that such neoplasms were often preceded by a long period of chronic irritation and inflammation. He postulated that chronic irritation and inflammation associated with ear marks may play a part in the development of skin cancer. Similar findings were reported by Ladd and Entwistle (1977) who observed that 39% of the tumours on the ear were associated with punch holes or ear marks.

The possibility of a co-carcinogenic agent involved in the aetiology of the disease has been suggested by a number of investigators (Carter 1958; Lloyd 1961; Vandegraaff 1976). The increased incidence of tumours observed by Lloyd (1961) was associated with an outbreak of photosensitization following ingestion of certain plants growing in the grazing area. Similar observations were reported by Carter (1958) who claimed that acute photosensitization with dermatitis was common in flocks with a high incidence of skin cancer. Photosensitization due to topical application of organo-phosphate chemicals for the treatment of cutaneous myiasis of the perineum has been implicated in the development of skin cancer in that region (Vandegraaff 1976).

Virological studies carried out on cultivated tumour material have so far revealed no evidence of viral involvement in the disease (Ladd and Entwistle 1977).

1.4 BOVINE OCULAR SQUAMOUS CELL CARCINOMA

Bovine ocular squamous cell carcinoma is an example of another malignant disease which has been attributed to the effect of

UV-irradiation from the sun. This disease has been reported to occur in a variety of animal species; morbidity is much higher in cattle than in other species, and the tumour is widely recognized as a particular disease problem in certain breeds of cattle (Russell *et al.*, 1956).

The age incidence of this tumour shows a similar trend to that of skin cancer in sheep. The disease occurs infrequently in cattle less than 5 years of age (Anderson *et al.*, 1957; Russell *et al.*, 1976); the incidence increases with age up to a peak at 8 years. The rate of metastasis of this tumour seems to be higher than that of ovine squamous cell carcinoma but it, too, spreads primarily by way of the lymphatic system (Russell *et al.*, 1956).

Many factors have been proposed as causes for eye cancer in cattle, but solar radiation has been postulated as being most important in the causation of the disease. Guilbert *et al.* (1948) have suggested that sunburn of nonpigmented orbital skin results in the development of ulcers and benign tumours, and malignant transformation follows with continued irritation. This is supported by the finding that "cancer eye" is particularly prevalent in areas where solar radiation is high and in cattle that lack eye pigmentation (Anderson *et al.*, 1956; French 1959; Anderson and Skinner 1961).

In addition to solar radiation, an influence of hereditary factors on the occurrence of ocular squamous carcinoma has been inferred by many investigators. Thus, although the disease has been reported to occur in many different breeds of cattle, it has been frequently stated that the Hereford breed is especially susceptible to ocular squamous carcinoma, (Frank 1943;

Woodward and Knapp 1950).

The possibility of viral involvement in the aetiology of bovine ocular carcinoma has been argued by some investigators (Nair and Sastry 1954; Russell*et al.*, 1956). Evidence to support this comes from studies by Taylor and Hanks (1969) who isolated infectious bovine rhinotracheitis virus (IBR) from eye cancer material.

Other factors have also been suspected to contribute to the disease. These include irritation caused by sand, insects, chemicals, thorny plants and photosensitization (Russell*et al.*, 1956).

1.5 MECHANISMS OF CARCINOGENESIS

1.5.1 Introduction

It is thought that the carcinogenic action of UV-light and of other physical and chemical agents is due to interactions which produce damage in the DNA of the target cells. It is well established that structural defects can be produced in DNA by UV-irradiation and this causes disruption of the continuity of the molecule. Other UV damage interferes with replication and transcription of DNA by causing changes in the hydrogen bonding. Single-strand breaks and cross-links between the DNA strands are induced by UV-light, but they occur only at high doses and their biological significance is questionable (Setlow *et al.*, 1968). Modification of the DNA bases which results in the formation of pyrimidine dimers is the most frequent lesion formed in DNA by UV-irradiation but again the significance of these alterations in carcinogenesis is not known.

1.5.2 *Mechanisms of DNA Repair*

Although a variety of agents can produce lesions in DNA, living plant and animal cells possess mechanisms which enable DNA damage to be corrected so as to re-establish the integrity of the molecule. Most of the early work on the mechanisms of DNA repair following UV-induced damage has been done using the bacterium *Eschericia coli (E. coli)*. In these cells three mechanisms of DNA repair have been identified; (1) Photoenzymatic repair, in which the damaged part of the molecule can be restored to a functional state *in situ*. This is accomplished by an enzymatic mechanism (Sutherland 1975). (2) Excision repair, which is a multi-enzymatic process in which the damaged part is removed and replaced by the insertion of the correct sequence of nucleotides necessary to restore the normal function of the original molecule (Cerutti 1974; Regan and Setlow 1974). (3) Post replication repair, in which the damage may remain in the DNA which is then repaired after replication by a complicated process which involves recombination of the damaged daughter DNA with paternal strands and *de novo* DNA synthesis (Rupp *et al.*, 1971; Lehmann 1974).

1.5.3 *Faulty DNA Repair and Carcinogenesis*

Although UV-induced damage to DNA can be repaired by a variety of mechanisms, cell mutations caused by UV-light, still occur. One possible explanation is that such mutations originate from errors in the repair process itself. Recently Witkin (1976), has shown that at least in *E. coli*, most of the repair procedures are free of error. Evidence however, has been provided for the existence of an enzyme system which seems to be involved in error-prone repair processes. The synthesis and operation of such an enzyme

system is normally suppressed in undamaged *E. coli* cells. However, when the bacterium is severely damaged by UV-light, the synthesis of the enzyme system is induced. This system seems to be involved in error-prone forms of repair which lead to the insertion of incorrect nucleotides into the damaged DNA strand and subsequently to mutation. Support for the above view linking error-prone DNA repair, mutagenesis and carcinogenesis has come from studies by Latarjet (1977), who found that the induction of skin cancer in mice by UV-light is strongly inhibited by caffeine (a specific inhibitor of error-prone repair process).

Other evidence for the role of unrepaired DNA damage in mutagenesis and possibly cancer induction is derived from the work of Cleaver (1968; 1969) on the pathogenesis of xeroderma pigmentosum. One important clinical finding in patients with xeroderma pigmentosum is the accelerated appearance of epithelial tumours of the skin at an early age. Such individuals have epithelial tumours about 10^3-10^4 times more frequently than are found in normal outdoor workers in tropical areas (Burnet 1978, Setlow 1978). Studies on cultured cells from patients with xeroderma pigmentosum have enabled these patients to be divided into groups which have different deficiencies in their DNA repair mechanisms. Cleaver (1968, 1969) reported that DNA repair in cultivated skin cells from xeroderma pigmentosum patients was defective following exposure to UV-light when compared to normal cells. This finding was confirmed by other investigators and was attributed to a defect in the excision-repair process (Setlow *et al.*, 1969; Lehmann *et al.*, 1975).

Xeroderma pigmentosum patients have also been found who have normal excision repair processes (Robbins *et al.*, 1974), but

the cells of these individuals have a slower rate of DNA synthesis compared to cells from normal individuals. In addition, the cells of these patients are defective in post-replication repair (Lehmann *et al.*, 1975, 1977). It is now thought that such defects in the DNA repair processes in xeroderma pigmentosum patients are partially responsible for their clinical symptoms and for their high incidence of skin tumours.

The evidence suggests that injury to DNA is somehow related to carcinogenesis. Epstein *et al.* (1971) suggested that the production of skin cancer by UV-light is initiated by the repair of DNA which while allowing the cell to survive, favours subsequent errors in DNA replication which result in mutagenesis and subsequently carcinogenesis. Alternatively, it was proposed that UV-light induced damage leads to neoplasia by promoting transformation by an oncogenic virus (Cleaver and Bootsma 1975).

With regard to the role of photosensitizing chemicals in carcinogenesis, it is believed that these chemicals result in augmentation of the primary reactions that underlie the sunburn response of the skin. A large amount of radiant energy is absorbed by the skin in the presence of the photosensitizing agents. This absorbed energy can cause cell damage directly or by creating covalent linkage between the sensitizing molecule and the pyrimidines in the cellular DNA. In addition, the photosensitizing molecules can transfer the absorbed energy and promote the formation of free radicals which cause damage to the cell membrane (Fitzpatrick and Pathak 1977). It is possible that these agents act in conjunction with UV-light to cause skin damage. Since skin damage and scar formation is often associated with neoplasia, it may be that photosensitizing substances act as co-carcinogens by

increasing the susceptibility of the affected areas to the harmful effects of UV-light.

1.6 CLINICAL OBSERVATIONS AND EXPERIMENTAL EVIDENCE SUGGESTIVE OF A HOST IMMUNE RESPONSE AGAINST TUMOURS

One of the functions of the immune system is to recognize and respond to foreign materials introduced into the body and to develop resistance against that foreign material. In the last forty years, evidence has been accumulating suggesting that the immune system is involved in controlling tumour growth. Support for this view is based on both clinical observations and experimental evidence (Baldwin and Price 1976b). The clinical observations include the following: (1) The demonstration that many tumours are infiltrated by large numbers of inflammatory cells. The presence of cellular infiltrates within the tumour is sometimes associated with a better prognosis (Black *et al.*, 1971; Underwood 1974; Dipaola *et al.*, 1977). (2) The increased reactivity of the regional lymph node in some tumour systems, which again is often associated with a better prognosis (Black and Speer 1958; Tsakraklides *et al.*, 1973, 1974; Berlinger *et al.*, 1976). (3) Spontaneous remissions are sometimes seen in patients with melanoma, neuroblastoma and renal cell carcinoma which are suggestive of an involvement of immune mechanisms (Sumner and Foraker 1960; Smith and Stehlin 1965; Everson and Cole 1966). (4) There is a well documented increase in the incidence of certain types of tumours in people with impaired immune reactivity, eg. in people with immuno-deficiency diseases (Gatti and Good 1971; Waldmann *et al.*, 1972; Penn 1974), in patients undergoing immunosuppressive therapy (Penn

and Starzl 1972; Penn 1975), and in aged people (Cairns 1975a, 1975b; Teasdale *et al.*, 1979).

Experimental evidence supporting the view that immune mechanisms are involved in controlling tumour growth, was first offered by Gross (1943), when he described the existence of tumour specific antigens. The demonstration of such antigens was crucial to sustain the concept of immune reactivity to tumours.

1.7 TUMOUR ANTIGENS

The existence of specific antigens on tumour cells induced by chemical, physical and viral agents has been established by the use of several different methods. The term tumour antigen has been used to describe two different classes of molecules (Alexander 1976a); those which are present on the tumour cells and can elicit an immunological reaction in the host, and those which are associated with the tumour and can be found on normal cells, and are only immunogenic in species other than the host. Based on their immunogenicity the antigens can define two distinct types of tumours; potentially immunogenic and weakly immunogenic tumours.

1.7.1 Potentially Immunogenic Tumours

This category includes: (1) tumours with unique tumour specific transplantation antigens (TSTA) arising from chemical induction; and (2) tumours which express antigens induced by either DNA or RNA oncogenic viruses. These tumours with few exceptions, express TSTA that can cause rejection when syngeneic animals are immunized with a potentially lethal dose of tumour cells.

A striking characteristic of the TSTA of chemically induced tumours of various histologic types, is the existence of individually distinct TSTA. Thus immunization with other tumours induced by the same chemical is usually ineffective in offering protection against subsequent challenge with the first tumour (Old *et al.*, 1962; Klein 1966; Baldwin 1973). In contrast to the diversity of TSTA expressed on chemically induced tumours, tumour cells induced by DNA and RNA oncogenic viruses possess antigens that cross-react with other tumours induced by the same virus. This group specific cross-reactivity extends across the species and the histologic type of neoplasms and strongly suggests viral induction or coding of TSTA (Habel 1961, 1962). Most of these neoplasms do not contain the replicating virus particles, and once the tumour is established, it does not depend upon virus proliferation for its continued growth.

It has been proposed that unique TSTA of chemically induced tumours are anomalously expressed H-2 or minor histocompatibility antigens. This was based on the finding that mice immunized with allogeneic tissue are sometimes protected against chemically induced syngeneic tumours (Invernizzi and Parmiani 1975). Anomalous reactions of H-2 antisera with chemically induced tumours have been also observed (Garrido *et al.*, 1976). Finally, it has been reported that unique TSTA of rat hepatomata resemble the major histocompatibility complex (MHC) antigens in molecular weight and amino acid composition (Bowen and Baldwin 1975).

1.7.2 *Weakly Immunogenic Tumours*

This category includes tumours with 2 classes of tumour associated antigens; oncofoetal antigens which are normally

expressed only by foetal tissue, and differentiation antigens which are normally expressed by certain differentiated adult cells. Oncofoetal antigens can be found on tumour cell membranes or they may represent tumour products secreted into the serum of tumour-bearing animals. Oncofoetal antigens are expressed by a wide range of tumour cells including those induced by chemicals and viruses (Baldwin *et al.*, 1974; Evans 1976). The expression of a foetal product by tumours was first demonstrated by Abelev *et al.* (1963), who showed that chemically induced rat hepatomata synthesize α-foetoprotein which is present in the serum of new-born mice but not of normal adults. Alpha-foetoproteins were subsequently identified in the serum of patients with hepatomata (Abelev 1971) and in those with germ cell tumours such as ovarian and testicular teratocarcinomas (Smith and O'Neill 1971; Abelev 1974). Alpha-foetoproteins are poorly immunogenic and only a low degree of protection has been obtained by immunization with foetal tissues (Baldwin *et al.*, 1974).

Another foetal product which was found to be associated with malignancy is termed carcinoembryonic antigen (CEA). Carcino-embryonic antigen was first described by Gold and Freedman (1965a, 1965b) as a substance that was present in adenocarcinomata of the human digestive tract and in the digestive organs of foetuses up to 6 months old, but not in normal adult tissues. These investigators suggested that these antigens represent cellular constituents which are repressed during the course of differentiation of the normal digestive system epithelium and reappear in the corresponding malignant cell by a process of dedifferentiation.

It was originally thought that both CEA and α-foetoprotein were specific for gastrointestinal and hepatic cell tumours

respectively. However, it was subsequently found that elevated levels of these tumour-associated products can be detected in other malignancies as well as in non-malignant diseases (Abelev 1974; Terry *et al.*, 1974). Accordingly, it is unlikely that the presence of these tumour products will be of great value in cancer diagnosis. However, monitoring the levels of α-foetoprotein and CEA in patients with hepatoma and colon carcinoma may be of clinical value since any variation in their concentrations is likely to reflect changes in the behaviour of the tumour.

Another group of tumour-associated antigens includes those antigens expressed only by certain normal differentiated adult cells, and tumour cells. The best example is the thymus leukaemia (TL) antigen of mice. TL antigen is structurally and functionally homologous to the H-2 antigens (Anundi *et al.*, 1975). In addition, tumours of lymphoid origin can express anomalously certain other normal cell-surface antigens. Some tumours were found to express antigens characteristic of both B and T cells, such as surface immunoglobulin and Thy-1 antigen (Schwartz *et al.*, 1977).

1.8 THE NATURE OF THE HOST RESPONSE

The discovery of the existence of tumour specific antigens led to a search for the effector mechanisms involved in the immune response to tumours. A wide variety of *in vitro* assays were developed to detect both humoral and cellular immune responsiveness directed against tumours. These will be briefly discussed here together with their relevance to the biology of the tumour. Immune responses to tumours may be antibody-mediated or cellular.

1.8.1 *Humoral Responses*

It has been generally held that anti-tumour antibodies play little or no role in controlling neoplastic growth. This proposition has been based on findings from both experimental and human tumour systems. Attempts to transfer passive protection against tumours with immune anti-tumour sera have been unsuccessful (Möller 1964; Baldwin and Barker 1967). Cytotoxic anti-tumour antibodies have been detected in some human tumours at an early stage of tumour growth and have been eluted from tumour cells of various histologic types *in vivo* (Irie *et al.*, 1974; Gupta and Morton 1975). These antibodies apparently have no protective effect for the host and consequently their role in controlling neoplastic growth has been discounted.

There are studies, however, that have indicated that antibodies may have a role in controlling tumour growth. In a murine leukaemia system, Old and Boyse (1964) found that the injection of immune sera inhibited tumour growth. Immune sera were also found to be effective in the treatment of tumours induced by Moloney virus (Fefer 1969; 1970). Moreover, in studies with induced primary sarcomata in rats, Alexander and co-workers (1970) found that the removal of antibodies from animals carrying subcutaneous implants increased the formation of metastases, while the passive transfer of these antibodies provided good protection against an intravenous injection of the tumour cells.

In some human tumour systems, a positive correlation between the presence of anti-tumour antibodies and the clinical course of the disease has been well documented. Malignant melanoma has attracted considerable interest because of reported instances of spontaneous regression (Sumner and Foraker 1960; Smith and Stehlin

1965) which suggests that immunological control may be involved. Studies with malignant melanoma have shown a clear correlation between the appearance of antibodies and the clinical course of the disease. Lewis *et al.* (1969) found that anti-melanoma antibodies were only detected in patients in which the disease was restricted and these antibodies disappeared when the disease became disseminated. One interpretation of this finding is that antibodies may be responsible for controlling the potential of tumours to metastasize. Once metastasis occurs, antibodies may become absorbed by circulating antigens and consequently are no longer detectable.

Recent studies have indicated that anti-tumour antibodies may be involved in protection against cancer, through an antibody-dependent cellular cytotoxic mechanism (ADCC). This is a cytotoxic reaction mediated by non-immune effector cells in the presence of specific antibody (MacLennan 1972; Perlmann *et al.*, 1972). Some aspects of this reaction have been characterized. The relevant antibodies were found to be of the IgG class, although IgM has also been shown to mediate ADCC in some situations (Lamon *et al.*, 1975). The exact nature of the cell involved is less clearly defined. It has been established, however, that the effector cell is not a T-lymphocyte (van Boxel *et al.*, 1972) and that it carries the Fc receptor for the immunoglobulin IgG (van Boxel *et al.*, 1973). The cytotoxic reaction is not complement dependent (van Boxel *et al.*, 1974) and does not involve phagocytosis (Greenberg *et al.*, 1973b). Cells from a variety of sources have been shown to be capable of mediating ADCC. These include adherent cells (Dennert and Lennox, 1973), and null cells (Greenberg *et al.*, 1973a).

Indirect evidence which is accumulating both from experimental animal models and clinical studies of human cancer suggests that ADCC may be an important defence mechanism *in vivo* against cancer. The Moloney sarcoma virus system (MSV) has been studied most extensively *in vivo* with regard to ADCC. MSV induces tumours which frequently undergo regression (Fefer *et al.*, 1967). Although immune T-cells are generally believed to be responsible for mediating the destruction of MSV-induced tumours, cells which are not T-cells have also been shown to be able to inhibit tumour growth *in vitro*, especially when obtained from animals in which the tumour has regressed (Lamon *et al.*, 1973, 1974). Moreover, it has been reported that sera from animals which have previously carried a tumour are capable of transferring immunity to MSV induced tumours. In general, sera collected from mice in which tumours have regressed, were most effective (Pearson *et al.*, 1973).

It was subsequently shown that sera, collected from mice in which tumours had regressed, were able to mediate ADCC *in vitro*, whereas sera taken from mice in which tumours were progressing had no detectable ADCC activity (Harada *et al.*, 1975). This correlation between the amount of tumour present and the level of ADCC has been demonstrated in other virus-induced tumours. Prather and Lausch (1976) found that with tumours caused by PARA-adenovirus in hamsters, ADCC activity was detected in the first week after tumour isografting but was no longer detectable at three weeks. Following surgical removal of the tumour, ADCC levels increased to a maximum at two weeks and persisted at a significant level for a month. Similar findings were obtained in the Gross lymphoma system in rats, where the appearance of ADCC

was closely associated with regression of the tumour (de Landazuri *et al.*, 1974).

Due to the extreme sensitivity of the ADCC assay in detecting low levels of antibodies (Möller and Svehag 1972; Kiessling and Klein 1973), it has been used extensively in human tumour systems. Data obtained so far have indicated that ADCC may be of prognostic value. Pearson *et al.* (1978) and Chan *et al.* (1979) found a correlation between ADCC titres to Epstein-Barr virus (EBV) membrane antigens and the rate of survival in patients with nasopharyngeal carcinoma. Patients with high ADCC titres at diagnosis survived significantly longer following therapy than those with low ADCC titres. Similar findings were obtained in patients with Burkitt's lymphoma (Granlund *et al.*, 1979; Pearson *et al.*, 1979). Patients with high ADCC titres showed partial or complete regression of lymphoma when treated by chemotherapy, while the lymphoma grew progressively in those with low titres. Other investigators have demonstrated a correlation between ADCC titres and the stage of the disease. Fäldt and Ankerst (1979) in their studies of patients with acute myelogenous leukaemia detected low levels of ADCC in all patients tested. They found that ADCC levels increased after therapy and were highest during clinical remission.

Although ADCC has been used extensively in detecting tumour antigens in a variety of experimental and human tumour systems, the validity of this assay in detecting tumour-specific antigens is questionable. Studies by different groups of investigators have shown that most of the cytotoxicity detected when lymphoid cells from cancer patients were cultivated with various tumour cell lines was related to ADCC (Pape *et al.*, 1977a). However, a

similar type of cytotoxicity was obtained when lymphocytes from healthy donors were cultivated with these tumour cell lines (Takasugi *et al.*, 1973; Pape *et al.*, 1977b). More studies by the same research group provided evidence for the existence of antibodies capable of mediating ADCC in the serum of both cancer patients (disease related antibodies) and healthy controls (natural antibodies) (Troye *et al.*, 1980a, 1980b). McCredie and MacDonald (1980) found that ADCC activity was the same in healthy individuals as it was in those with benign diseases. It was decreased in patients with advanced cancer but also in patients with septicaemia

1.8.2 *Cellular Responses*

There is a substantial body of evidence which shows that lymphoid cells from tumour-bearing animals or from cancer patients are toxic for tumour cells *in vitro*. Furthermore, adoptive transfer experiments in which various cell populations have been shown to offer protection against subsequent challenge with specific tumours suggest that the cellular response to a tumour is important in controlling its growth. Techniques which identify cells according to their surface markers and functional properties have implicated a variety of cells of lymphoreticular origin in tumour destruction.

(a) Thymus-derived Lymphocytes (T-Cells)

The role of T-cells in controlling tumour growth has been determined mainly from studies with virally and chemically induced tumours. In tumours induced by Moloney sarcoma virus (MSV), alterations in T-cell reactivities were detected early after

infection with the virus, when the tumour was developed and again at the time it was regressing (Lamon *et al.*, 1973). Similarly, Leclerc *et al.* (1973) and Plata and Levy (1974) demonstrated T-cell-mediated cytotoxicity against MSV induced tumours. Cytotoxicity could be detected early after virus infection; cytotoxic activity reached a peak around the time when the tumour had begun to regress. In contrast, cytotoxic activity disappeared within a few days in younger mice which were unable to reject their tumours. The killing of tumour cells by T-lymphocytes has also been implicated in plasmacytoma and in EL-4 leukaemia in the mouse (Rouse *et al.*, 1972, Wagner and Röllinghoff 1973). Rouse *et al.* (1972) have demonstrated *in vivo* that cells capable of transferring immunity to syngeneic murine plasma cell tumours were sensitive to treatment by anti-θ serum and complement.

In man the role of T-cells in controlling neoplastic growth is conjectural. Only a few reports have indicated that the cytotoxicity detected against tumour cells *in vitro* was mediated by T-cells. Such reports include observations on melanoma (Wybran *et al.*, 1974), lung cancer (Vose *et al.*, 1978) and bladder carcinoma (Troye *et al.*, 1980a). However, in some of these studies, there was only suggestive evidence that the reaction is mediated by T-cells and consequently tumour cell death being mediated by natural killer cells (NK) remains a possibility.

Another assay which has been used extensively in studies of cellular responses to antigens on transformed human cells is the blastogenesis assay. The responsiveness of lymphocytes to tumour antigens detected using this assay, has been shown to be mediated by T-cells, at least in the mouse system. Positive

responses were obtained with lymphocytes exposed to Burkitt's lymphoma (Stjernswärd *et al.*, 1970) human leukaemia (Gutterman *et al.*, 1972) and other lymphoblastoid cell lines (Svedmyr *et al.*, 1974). In solid human tumours the significance of the blastogenesis assay has been more difficult to assess. Low positive responses were obtained with melanoma cells (Nagel *et al.*, 1971), sarcoma cells (Vànky *et al.*, 1971), cells from breast tumours (Stjernswärd *et al.*, 1973) and from other cancers (Vànky *et al.*, 1975). The difficulty in demonstrating blastogenic responses with solid tumour cells may be a function of the stage of the disease at which the test is done. It has been shown by Lamon and co-workers (1973, 1974), that different cell populations are involved in the host's response to tumour cells during different stages of the disease. Alternatively, the difficulty in demonstrating blastogenesis may be related to the nature of the antigen on the tumour cells.

(b) Macrophage Responses

Evidence from experimental tumour models has identified macrophages as important anti-tumour effector cells. Macrophages have been shown to inhibit the growth of tumour cells and also to kill them *in vitro* by a non-phagocytic mechanism that requires contact between the macrophages and the tumour cells. Two types of effector macrophages have been described, the non-specifically "activated" macrophages and the specifically "armed" macrophages. The "activated" macrophages were described by Hibbs *et al.* (1972) as a non-specific cytotoxic cell from animals infected with parasitic organisms. A number of microbes and microbial products have been shown to activate macrophages and although the cytotoxicity exhibited by these cells is non-specific in an

immunological sense, it is nevertheless selective. Thus cytotoxicity is most readily detected against transformed cells, but not against normal cells (Hibbs 1973; Kaplan *et al.*, 1974; Meltzer *et al.*, 1975; Mantovani *et al.*, 1979). The armed macrophages are cells that can participate in immunologically specific reactions by acting in co-operation with immune lymphocytes (Evans and Alexander 1970). Macrophages can be armed by contact with either immune lymphoid cells or with cell-free supernatant from cultures of immune T-cells incubated with antigen (Evans and Alexander 1972a and b). Macrophages so armed acquire the ability to kill the specific target. The difference between "activated" and "armed" macrophages lies in the method by which they are generated and the specificity of the cytotoxic reaction (Krahenbuhl and Remington 1974). The two terms are not mutually exclusive, however, since "armed" macrophages, following specific interaction with antigen become activated and kill in both a specific as well as non-specific way.

Based on results derived from experimental tumour models, it has been suggested that macrophages may play a role not only in the regulation of tumour growth but also in controlling its metastatic spread (Evans 1972; Zarling and Tevethia 1973). A clear correlation has been demonstrated between the content of macrophages in the primary tumour, the immunogenicity of the tumour and its metastatic potential (Eccles and Alexander 1974). In these studies with a chemically-induced fibrosarcoma in rats, Eccles and Alexander (1974) found that tumours with a high content of macrophages were highly immunogenic and showed little tendency to metastasize. Similarly Wood and Gillespie (1975) found that when macrophages were depleted from tumour inocula, this resulted

in an increase in the metastatic potential of these tumours and the recipient animals had lower rates of survival. In addition the administration of activated macrophages has been reported to prolong the survival of mice inoculated with tumour (van Loveren and den Otter 1974).

The mechanism(s) by which "armed" or "activated" macrophages inhibit tumour growth and kill tumour cells is not clear. Although Granger and Weiser (1966) demonstrated large amounts of immunoglobulins on the surface of cytotoxic macrophages this finding provides no conclusive evidence for the involvement of cytophilic antibodies in the cytotoxic process since inhibition of tumour growth was found to be unaffected by anti-Ig antiserum (Evans and Alexander 1972c). Recently, Currie (1978) reported that activated macrophages exert their effect on tumour growth through the release of arginase which cleaves the essential amino acid arginine.

Macrophages appear therefore to play an important part in the body's defence against neoplastic growth. The fact that "activated" macrophages have selective toxicity against transformed cells *in vitro* suggests that they may be important in providing an early surveillance mechanism which precedes or parallels immune recognition (Hibbs 1974; Alexander 1976b).

(c) Natural Killer Cell Responses (NK)

Natural killer cells are lymphoid cells which are spontaneously cytotoxic *in vitro* to a wide variety of cultivated tumour cell lines. As they occur naturally in animals which have not been previously sensitized and because they have the ability to kill transformed cells selectively, it has been postulated that

these cells act *in vivo* as a mechanism of surveillance against potentially neoplastic cells (Haller *et al.*, 1977; Kiessling and Wigzell 1979). Evidence to support this proposition comes mainly from observations in mice. A positive correlation has been observed between NK cell activity *in vitro* and tumour resistance *in vivo* (Zarling *et al.*, 1975; Minato *et al.*, 1979); eg. mouse strains with a high incidence of leukaemia have been found to express little or no NK activity. Also, mice genetically deficient in NK activity (*Beige* mice), have poor resistance to the growth of small doses of a variant of B16 melanoma, and large numbers of metastases form following the inoculation of tumour cells (Talmadge *et al.*, 1980). Furthermore, mice which have low levels of NK cells can be made resistant to tumours by the transfer of bone-marrow cells from strains that have high levels of NK cells (Haller *et al.*, 1977).

Several extraneous agents including bacterial adjuvants (Wolfe *et al.*, 1976) and viruses (Welsh and Zinkernagel 1977) produce increased NK activity *in vivo* and at the same time this increases resistance to the growth of transplantable tumours. Since several of these agents are also known to induce the production of Interferon, the possibility has been raised that Interferon may be the mediator or regulator by which NK activity is expressed and this leads, in turn, to an increased resistance to tumours. Support for this idea rests on results of experiments in mice which showed that, after the injection of substances which induce the production of Interferon, similar kinetics were demonstrated for the appearance of both NK cells and Interferon. Also the injection of mice with anti-Interferon globulin inhibited the increase in NK activity *in vivo* that usually follows the

injection of Tilorone (an inducer of Interferon). The injection of mouse Interferon preparations has also been shown to cause an increase in NK activity *in vivo* (Gidlund *et al.*, 1978).

Interferon not only regulates the activity of lymphoid cells but it is also produced by these cells. Trinchieri *et al.* (1977) have shown that incubation of human lymphocytes with various established cell lines, resulted in the production of Interferon, and this, in turn, produced a concomitant increase in NK activity. These findings were confirmed by Saksela *et al.* (1979) who showed furthermore, that any increase in the production of NK cells can be prevented by treatment with sheep anti-Interferon antiserum.

The mechanism by which Interferon acts to produce an increase in NK activity is not clear. Experiments using both human and mouse cells suggest that it may act by inducing pre-NK cells to differentiate into mature killer cells. Timonen *et al.* (1979a, 1979b) used an absorption-elution procedure to isolate and identify human NK cells from a population of lymphoid cells. They found that NK cytotoxic activity in the adherent fraction could not be augmented either by Interferon or by incubating these cells with the appropriate tumour cell line. However, NK cell activity could be increased in the other non-adherent cell fraction by adding Interferon to it. These findings were similar to those described in the rat by Oehler and Herberman (1978) who were able to eliminate mature NK cells by various procedures and to show also that inducers of Interferon production were able to restore NK activity in the remaining cell population in a few hours. These investigators suggested that Interferon is able to transform a non-cytotoxic pre NK-cell population to a cytotoxic one.

Despite the fact that there has been an extensive effort to characterize NK cells, their precise nature remains uncertain. Evidence from mice indicates that these cells arise *de novo* in the bone marrow (Haller and Wigzell 1977), that they develop independent of thymic influence (Herberman *et al.*, 1975a) and that they lack the characteristics of mature macrophages, B-cells or T-cells (Herberman *et al.*, 1975b). Most reports agree that the majority of NK cells possess Fc receptor (Herberman *et al.*, 1977; Kay *et al.*, 1977).

1.9 MECHANISMS OF TUMOUR ESCAPE

Despite the existence of all these anti-tumour mechanisms detected by various *in vitro* and *in vivo* assays, tumours often grow and kill their hosts. The efficacy of the cellular immune response *in vivo* may be diminished by the presence of humoral factors which interfere with existing host immunity and consequently provide the tumour with an escape route from host immunological control. This concept was originally developed by the Hellströms (1974) to explain their finding that sera from animals with a growing tumour can prevent immune lymphocytes from killing tumour cells with the same TSTA. A strong correlation was found between the presence of blocking factors in the serum of patients and the clinical course of the disease. Patients who had progressive disease more frequently had blocking factors in their sera than did patients who were tumour free at the time of testing.

The ability of sera from tumour-bearing patients to block cell mediated cytotoxicity to tumours was attributed originally to

blocking antibodies. It was thought that these antibodies enhance tumour growth by coating the antigenic sites on the tumour cells, thus protecting them from attack by immune lymphoid cells. Later findings by Sjögren *et al.* (1971; 1972) however, suggested that the blocking effect was due to antigen-antibody complexes, rather than antibody alone. These complexes can vary in their ratio of antigen to antibody and may have either antibody excess (Baldwin *et al.*, 1972) or antigen excess (Thomson *et al.*, 1973).

A different type of interference to the cytotoxic activity of lymphoid cells, termed "inhibition", has also been described. This type of blocking is due to circulating tumour antigens, which can interact with antigen-specific receptors on immune lymphocytes and render the cells non-cytotoxic. Baldwin and co-workers found that the blocking effect of tumour extracts on cell-mediated cytotoxicity *in vitro* occurs when the extracts were added either to the effector cells prior to the assay and then removed or when the extracts were left with the effector and target cells for the duration of the assay (Baldwin and Price 1976a). Currie and Basham (1972) reported that lymphocyte-bound, tumour-associated antigens were detected in cancer patients. When these antigens were eluted from the surface of lymphocytes, the cytotoxicity of these cells against the tumour cells was augmented.

The host-tumour interaction is complicated further by the discovery of serum factors that can neutralize the effect of blocking factors. Hellström *et al.* (1971) found that sera from patients free of disease rarely had any blocking activity. Moreover, when these sera were mixed with sera that manifested blocking activity the blocking effect was no longer detected.

Such sera were termed "unblocking" sera. Similar findings on this unblocking effect of sera have been demonstrated in other tumour systems (Robins and Baldwin 1974; Prather and Lausch 1977).

Although the blocking theory provides an explanation of how established tumours can escape destruction by the immune system, it is inadequate to explain why the host's immune system fails to destroy a nascent tumour. A number of possible mechanisms have been advanced to account for the "early" escape of tumours. Old and Boyse (1964) proposed that a nascent tumour may not initially stimulate the immune response (sneaking through) and by the time sufficient tumour is present to cause stimulation of the host's immune system, the established tumour may grow at a rate beyond host control. The concept of immunological enhancement provides an alternative hypothesis to explain why the immune system fails to destroy tumours, (Kaliss 1962). According to this concept, low levels of circulating antibodies, which are specific for the tumour, enhance tumour growth by binding to the antigenic sites on the tumour cell and thus preventing attack by immune cells. Finally, it has been proposed by Prehn (1971, 1977) that the immune system may play a dual role in the control of tumours. A weak immune response against tumour-specific antigens will stimulate tumour growth whereas a strong immune response could lead to tumour rejection. According to this proposal, nascent tumours stimulate a weak response which results in enhancing their growth.

It is apparent from the previous discussion on the involvement of the immune response in defence against cancer that the tumour-host relationship is extremely complex. A wide range of lymphoid cells and their products are involved in the

body's reaction to tumours and each population of cells may mediate its anti-tumour effect through a different mechanism. The relative importance of each cell type in any host-tumour interaction is greatly influenced by the nature of the antigen on the tumour cell, the clinical behaviour of the tumour (Miller and Heppner 1979), its mode of interaction with the host's immune system and finally the stage of tumour growth and differentiation.

1.10 SCOPE OF THE THESIS

The experiments described in this thesis were designed to elucidate certain aspects of the immunobiology of an ovine squamous cell carcinoma. The approach taken was to examine certain characteristics of the tumour cell populations as well as the host's response to its own tumour.

Methods were first established for the successful cultivation of pure populations of epithelial tumour cells *in vitro*. These cells were then examined by both light and electron microscopy and their morphological characteristics described. The growth rates of the various cultivated tumour cell lines were measured *in vitro*, and the ability of these cultivated tumour cells to form tumours in immunosuppressed hosts was examined. Further characterization of the tumour cells was done by examining their interaction with allogeneic lymphocytes both *in vitro* and *in vivo* and the capacity of the tumour cell lines to stimulate and suppress an allogeneic response was investigated.

Finally, studies were done to examine the immune response of a tumour-bearing sheep to autochthonous tumour cells using a cannulated isolated lymph node technique. This was done while

the primary tumour remained *in situ* and following resection of the tumour. A comparison between the immune response of the regional and a distant lymph node to challenge with autochthonous tumour cells was also carried out and the responses in these two sites compared.

CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS AND SURGICAL PROCEDURES

2.1.1 Experimental Animals

Merino sheep were used for all the allogeneic experiments. Tumour-bearing sheep, either Merino or Merino-Border Leicester cross, were collected from private properties in the district of Goondiwindi, Queensland. All sheep were kept indoors or in small paddocks for most of the time. They were transferred to metabolism cages a few days before the operation. This was done to accustom the sheep to the conditions in which the subsequent experiment would take place. While in metabolism cages, the sheep were fed lucerne chaff and grain oats and given water *ad libitum*. Sheep were deprived of food and water 24 hours before surgery.

The nude mice which were used were either BALB/c or CBA/H-BALB/c Fl (regular). Mice were bred and maintained in a pathogen-free environment in the John Curtin School of Medical Research Animal Breeding Establishment (ABE).

2.1.2 Anaesthesia

For induction of anaesthesia in sheep, thiopentone sodium (Intraval Sodium) was injected intravenously at a dose of 15-25mg/kg of body weight for normal sheep. For tumour-bearing sheep this dose was decreased to 10-18mg/kg of body weight because they were often in poor health. After the induction of anaesthesia, a Magill's tube was inserted into the trachea and inflated. The sheep was then transferred to the operating table and connected to a Boyle's closed circuit anaesthetic apparatus. Anaesthesia was maintained during the operation with

Halothane (Fluothane, ICI Ltd) and oxygen.

2.1.3 *Cannulation of lymphatic vessels*

(a) Cannulation of the afferent lymphatic of the popliteal lymph node

The afferent lymphatics draining to the popliteal lymph node are disposed on the anterior and posterior aspects of the saphenous vein. There are usually 3 or 4 lymphatics in the fascia close to the vein on each side. An incision was made in the skin over the vein just below the hock and the edges of the incision retracted. The fascia was separated from the vein on each side and the afferent lymphatics identified. A loose thread was passed around the fascia embracing all the lymphatics. The thread was tied loosely and the leg massaged to cause the lymphatics to swell. The largest and most appropriate lymphatic was selected and cleaned of adherent connective tissue for about 1 cm. The cannulation site was chosen about 2 cm below the occluding tie. A fine thread was passed around the lymphatic and tied to allow tension to be applied to the lymphatic and stretch it. A second fine tie was passed provisionally around the lymphatic and left loose. The lymphatic was cut with fine scissors and a bevelled plastic tube passed into the lymphatic and carried forward to the embracing tie. This tie was then loosened and the tube passed further along the duct towards the node. When the best position for the tube was established it was tied into the duct and secured by one or two stay sutures. The wound was dusted with penicillin and the skin incision closed with silk sutures.

(b) Cannulation of the efferent lymphatic of the popliteal lymph node

The efferent lymph duct from the popliteal node was approached through a skin incision that extended for about 8-10 cm vertically 4 cm below the sciatic tuber. The incision was carried through the intermuscular sheaths of the biceps and semimembranosus muscles. These two muscles were retracted and the posterior femoral blood vessels ligated and severed. The fascia around the femoral vein was examined and the popliteal lymphatic identified and tied off. The lymphatic was cleaned of adherent connective tissue along a length of about 3 cm and an occluding tie passed around the duct cranially. The ligated duct soon became swollen and a further non-occluding tie of fine silk was passed around it. Tension was applied to the duct with the cranial tie and the duct opened up with fine scissors. A plastic tube of the appropriate diameter was passed into the duct against the direction of lymph flow and adjusted to lie along the direction of the duct. Care was taken to ensure that the tip of the cannula was clear of any valves in the duct and that the lymph flow was unobstructed. The tubing was led out through a stab wound in the skin below the sciatic tuber and a silk purse-string suture inserted around the tubing where it emerged through the skin.

(c) Cannulation of the afferent and efferent lymphatics of the cervical lymph nodes and the regional nodes associated with tumours.

Tumours located on the ears and on the face and nose drain to regional nodes such as the parotid, the cranial cervical and the sub-mandibular nodes. The appropriate nodes were identified by the injection of a solution of T-1824 into the tumour and the draining lymphatics and the regional lymph node identified. The

appropriate afferent and efferent lymphatics were cleaned and cannulated by the techniques described for the popliteal lymphatics.

The deep cervical lymph duct was cannulated in some sheep. This is a large lymphatic that runs in the fascial sheath incorporating the sympathetic nerve trunk and the carotid artery. This duct was cannulated against the direction of lymph flow.

Lymph was collected into plastic bottles which were attached to plastic bottle holders sutured to the skin of the sheep at a site adjacent to where the tubing was led out of the skin.

2.1.4 *Lymphadenectomy*

Lymph nodes were removed by surgical excision. Popliteal nodes and cervical nodes which had received injections of cultivated tumour cells or which may have retained metastatic tumour cells were located and removed for fixation and histological study. In some cases the nodes were macerated to obtain cells.

2.2 PHYSIOLOGICAL SOLUTIONS, BUFFERS, FIXATIVES AND OTHER SOLUTIONS

2.2.1 *Physiological Solutions*

NaCl solution (Normal saline)

One litre of this solution contained 9.0 g of NaCl. The solution was sterilized by autoclaving for 20 min.

Hanks' balanced salt solution (HBSS)

A litre of this solution contained 8.0 g NaCl, 0.84 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.1 g $MgCl_2.6\ H_2O$, 0.14 g $CaCl_2$, 0.06 g Na_2HPO_4, 0.06 g KH_2PO_4, 1.0 g glucose, 20 mg phenol red. The solution was sterilized by autoclaving at 112°C for 20 min. The pH was adjusted to 7.4 with 5.0% $NaHCO_3$ solution.

Bovine serum albumin (BSA, 1%)

This solution was prepared by dissolving 1.0 g of bovine serum albumin (BSA) in 100 ml of Hanks' solution. The pH was adjusted to 7.4 with 5% $NaHCO_3$ solution.

Dulbecco's modified Eagle medium (H-16)

10 g of the powdered medium (Cat. H-16, Grand Island Biological Company, Grand Island, N.Y., USA) was dissolved in 926 ml of double distilled water and 74 ml of 5% $NaHCO_3$ was added to the solution. The pH of the solution was adjusted to 7.4 using CO_2 and the solution was sterilized by membrane filtration (Millipore filter, .22 µ membrane). Two ml of antibiotics (penicillin 100 u/ml, streptomycin 100 µg/ml, neomycin 100 µg/ml, PSN) was added to the medium which was then transferred to 150 ml bottles and stored at $4^{o}C$.

2.2.2 *Buffer Solutions*

Hepes buffer (1.0M, pH 7.4)

Stock solution (1 M) of this buffer was prepared by dissolving 23.8 g of Hepes (Sigma Chemical Company, USA) in 80 ml of distilled water. The pH was adjusted to 7.4 with 6N NaOH and the solution was made up to 100 ml. The solution was sterilized by autoclaving at $121^{o}C$ for 20 min. One ml of this solution was added to 100 ml of H-16 medium before use.

Acetate buffer (.05M,pH 5.0)

Glacial acetic acid (2.9 ml) was diluted in 800 ml of water. The pH was adjusted to 5.0 using 6 M NaOH, and the solution was made up to one litre.

Phosphate buffered saline (PBS)

A 10-times concentrated solution was prepared by dissolving 34.5 g $NaH_2PO_4.2H_2O$ and 81.8 g NaCl in about 800 ml of water. The pH was adjusted to 7.5 with 6M NaOH and the solution made up to one litre. The solution was diluted 10-times before use.

2.2.3 *Fixatives*

Buffered formaldehyde solution (4%)

This solution was prepared by dissolving 40 g of para-formaldehyde in one litre of PBS by boiling. The solution was then cooled to room temperature and the pH adjusted to 7.5 with PBS stock solution.

Ethyl-ether (1:1)

This solution was prepared by mixing equal volumes of 95% ethyl alcohol and ether. Slides to be stained with Papanicolaou stain for keratin (Thompson and Hunt 1966) were fixed for 30 min in this solution before staining.

2.2.4 *Stains*

Peroxidase staining solution

This solution was prepared by dissolving 4 mg of 3-amino-9-ethyl carbazole in 1 ml of dimethylformamide. Acetate buffer, 19 ml, (.05M, pH 5.0) was added to the above solution. Just before use, 3-4 drops of 3% H_2O_2 were added to the above mixture.

Leishman's staining solution (0.15%)

This solution was prepared by dissolving 0.15 g of the powder in 100 ml of absolute methanol. The solution was filtered and stored at room temperature. For staining slides with

cytocentrifuge preparations of efferent lymphocytes, 1 ml of this solution was added to each slide and left for 3 min. Two ml of distilled water were added to each slide and left for 10 min. Slides were then rinsed with tap water and left to dry.

Trypan blue (0.5%)

The solution was prepared by dissolving 0.5 g of the powder in 100 ml of PBS. The solution was filtered and stored at room temperatures. Before use the solution was diluted 4 times in PBS. The final concentration of the dye was 0.125%.

2.2.5 *Other Solutions*

Ficoll-isopaque

Ficoll solution (8%) was prepared by dissolving 8 g of ficoll (Ficoll 400, Pharmacia, Fine Chemicals AB, Uppsala, Sweden) in 80 ml of distilled water before making it up to 100 ml. Sodium metrizoate, (30 ml of 32.8% W/V) was added to 70 ml of 8% ficoll solution. The solution was protected from light with foil wrapping and sterilized by autoclaving at 121^{o}C for 20 min. The specific gravity of the final solution was 1.077 g/ml.

Heparin solution

This solution was prepared by dissolving the contents of a vial of powdered heparin (100,000 units) in 66.6 ml of 0.9% NaCl. This gave a final concentration of 1500 units/ml. The solution was sterilized by membrane filtration (Millipore filter, .45µ membrane) and was used at a final concentration of 30 units/ml of blood.

Trypsin diluent (0.5%)

One litre of this solution contained 8 g NaCl, 4 g KCL, 3.75 g $Na_2HPO_4.2H_2O$, 3 g $KHPO_4$. The solution was sterilized by autoclaving at 121°C for 20 min.

Trypsin solution (10%)

One litre of this solution contained 8 g NaCl, 0.4 g KCl, 0.78 g $Na_2HPO_4.2H_2O$, 0.06 g KH_2PO_4, 50 g glucose, 100 g trypsin (Trypsin 1:250 DIFCO Laboratories, Detroit, Michigan, USA). The solution was centrifuged at 27,578 g for 60 min. The supernatant was collected, sterilized by membrane filtration, dispersed into 5 ml vials and stored frozen.

Trypsin solution (0.5%)

This solution was prepared by diluting 5 ml of 10% trypsin stock solution with 95 ml of trypsin diluent. The pH was adjusted to 7.4 with 5% $NaHCO_3$. The solution was sterilized by membrane filtration and was transferred to 5 ml vials and stored frozen until used. The solution was used at a final concentration of 0.25%.

Versene (EDTA, 0.2%)

The solution was prepared by dissolving 0.2 g of EDTA (Ethylene diamine tetra-acetic acid disodium salt, AJAX Chemicals, Sydney, Australia) in 100 ml of trypsin diluent. The pH was adjusted to 7.2 with 5% $NaHCO_3$. The solution was sterilized by autoclaving at 121°C for 20 min and was stored at 4°C. The solution was used at a final concentration of 0.1%.

2.3 TUMOUR CELL LINES

2.3.1 Maintenance

All established tumour cell lines were maintained in tissue culture flasks of either 25 cm^2 or 75 cm^2 growth area (Falcon plastics, Div., Becton Dickinson and Co. Oxnard, California, USA). Tumour cells were fed with Dulbecco's modified Eagle medium (H-16, Gibco) supplemented with 10% V/V heat-inactivated foetal calf serum (FCS, Gibco, USA) containing PSN antibiotics (medium containing FCS and PSN will be referred to as "complete medium"). Cultures were gassed using 10% CO_2 in air and were incubated at 37^{o}C. When the cells became confluent, they were subcultured.

2.3.2 Subculture

Cell monolayers were washed twice with trypsin diluent. This procedure removed all the foetal calf serum which inhibits trypsin activity. Two ml of trypsin-versene solution (final concentrations 0.25% and 0.1% respectively) were added to small 25 cm^2 flasks or 4 ml to large 75 cm^2 flasks. Tissue culture flasks were then incubated at 37^{o}C for 10 min after which the trypsin was removed and the flasks tapped to detach the cells mechanically. Cells were resuspended in complete medium, washed twice by centrifugation at 300 g for 10 min and resuspended in complete medium. The viable cells were counted in a haemocytometer using Trypan blue and the cell number was adjusted to the required concentration (10^5 viable cells/ml). Five or 15 ml of the cell suspension were transferred to 25 cm^2 and 75 cm^2 tissue culture flasks respectively.

2.3.3 *Cryopreservation*

(a) Freezing

Tumour cells were harvested from tissue culture flasks using trypsin-versene solution as described in the previous section. Cells were washed twice in complete medium, counted using trypan blue and resuspended at a concentration of 2×10^6 viable cells/ml in H-16 + 30% FCS and kept on ice. An equal volume of cold H-16 + 30% FCS + 20% DMSO (Dimethylsulfoxide) was added to the cell suspension, and one ml portions of cell suspension were transferred into 3 ml glass vials which were placed on dry ice for 30 min. The vials were transferred to the nitrogen freezer. The final composition of the freezing medium was 60% H-16, 30% FCS and 10% DMSO.

(b) Thawing

To thaw the cells, the frozen vials were removed from the nitrogen freezer and immersed immediately in a $37^{\circ}C$ water bath. Just before the cells were completely thawed, vials were transferred from the water bath and placed on ice for 2 min. The cell suspension in the vials was diluted very slowly by the drop-wise addition of 1-2 drops of cold H-16 + 30% FCS every 10 seconds, until a volume of 3 ml was reached. Cells were then transferred into cold 15 ml centrifuge tubes and one ml of H-16 + 30% FCS was added to the suspension which was left on ice for an additional 5 min. This procedure was repeated until a final volume of 10 ml was reached and at this stage the cells were left on ice for 15 min. Cells were washed twice in H-16 + 30% FCS by centrifugation at 120 g for 10 min. They were then counted, resuspended in complete medium at a concentration of 10^5 viable cells/ml and transferred into 25 cm^2 flasks at a cell density of 5×10^5 cells/flask.

2.4 LYMPHOCYTE PREPARATIONS

2.4.1 Separation of Peripheral Blood Leucocytes

Peripheral blood leucocytes were separated from whole blood according to the method originally described by Böyum (1976) with some modifications. Blood was collected from the jugular vein of sheep using a 60 ml syringe fitted with an 18-gauge needle and containing heparin for a concentration of 30 units/ml blood. Blood samples were transferred into large 45 ml tubes and were centrifuged at 1000 g for 15-20 min at 4°C. The buffy coat formed by this centrifugation was collected with a Pasteur pipette and diluted in twice its volume with Hanks' solution containing EDTA (final concentration of EDTA in Hanks' was .075%). Seven ml of the diluted buffy coat was then layered on 3 ml of ficoll-isopaque solution. The tubes were then centrifuged at 600 g for 40 min. The interface layer containing mononuclear cells was aspirated with a Pasteur pipette and diluted in Hanks'/EDTA solution. Cells were washed twice in Hanks'/EDTA by centrifugation at 200 g for 10 min each. This was followed by two further washes in Hanks' solution. After the final washing the cells were resuspended in complete medium, counted using Trypan blue and the cell number adjusted to the required concentration.

2.4.2 Separation of lymph node lymphocytes

Cell suspensions from lymph nodes were prepared according to the method of Lafferty *et al.* (1974) with some modifications. Cervical and popliteal lymph nodes were removed aseptically and placed in HBSS containing PSN. Lymph nodes were trimmed of fat, cut into several pieces and passed through a 400 stainless steel

mesh into fresh HBSS. The cell suspension was transferred into a centrifuge tube and allowed to settle for 2 min to remove debris and cell clumps. The supernatant, consisting of a suspension of single cells, was then transferred to another tube and centrifuged at 300 g for 10 min. The cell pellet was washed twice in HBSS. After the final washing, the cells were resuspended in complete medium, counted and the cell number was adjusted to the required concentration.

2.5 IRRADIATION OF STIMULATOR CELLS

Tumour cells were harvested from tissue culture flasks, washed twice and resuspended at a concentration of 1×10^6/ml in complete medium. Leukocytes, either efferent lymph cells or cells separated from peripheral blood, were washed 3 times and resuspended at a concentration of 1×10^7 cells/ml in complete medium. Cells were irradiated using a cobalt (^{60}Co) source (CSIRO, Division of Plant Industry) at a dose of 1200 rads for lymphocytes and 7000 rads for tumour cells.

2.6 IN VITRO ASSAYS FOR CELL-MEDIATED IMMUNITY

2.6.1 The Mixed Lymphocyte Culture

The mixed lymphocyte cultures and the lymphocyte tumour cell cultures were set up in sterile disposable plastic tissue culture trays using cell concentrations as mentioned in individual experiments. For experiments in which only ^{3}H-thymidine uptake was to be determined, equal volumes (0.1 ml) of the stimulator (either tumour cells or lymphocytes) and the responder cell suspensions were mixed in 6 mm diameter wells in 96 well trays

(Linbro Model FB-96-TC Multidish Disposo-trays, Linbro Chemical Company, New Haven, Conn., USA). For experiments in which both ^{3}H-thymidine uptake and cytotoxic cell generation were to be assayed, equal volumes (1.0 ml) of the stimulator and responder cell suspensions were mixed in 16 mm diameter wells in 24 well trays (Linbro Model FB-16-TC Multidish Disposo-trays, Linbro Chemical Co., USA). The control for the mixed lymphocyte culture consisted of cultivating together equal concentrations (5×10^{6}/ml) of responding lymphocytes and γ-irradiated responding lymphocytes. As a control for the lymphocyte-tumour cell interaction, both lymphocytes and γ-irradiated tumour cells were cultivated separately at the appropriate concentration and the uptake of thymidine in these cultures was determined. Stimulation was considered to have occurred when the mean value for thymidine uptake in cultures containing tumour cells in the presence of lymphocytes plus two standard errors was higher than the sum of the values for thymidine uptake obtained from the cultures in which each cell type was cultured alone. All cells prepared for the mixed lymphocyte cultures were suspended in the complete medium containing 10 mM Hepes and 10^{-4} M 2-mercaptoethanol (2ME). All assays were performed in triplicate wells. Trays were placed in airtight boxes, gassed with humidified 10% CO_2, 7% O_2 and 83% N_2 and maintained at 37^{o}C for the required period of time.

(a) ^{3}H-thymidine uptake

^{3}H-thymidine uptake was assayed at various time intervals after the cultures were initiated, in 96 well trays. For cultures set up in 24 well trays, 0.2 ml portions were transferred into wells of 96 well trays. ^{3}H-thymidine (Methyl ^{3}H-thymidine, S.A.

5 µCi/mmol, The Radiochemical Centre, Amersham, U.K.) was diluted 50 µCi/ml in Hanks' solution and 25 µl of this solution was added to each well. Trays were then returned to gas boxes and incubated at 37°C for 5 hr. Following incubation the contents of individual wells were harvested onto glass filter strips (Cat. No. 934-AH, Reeve Angel, Clifton, New Jersey, USA) with a Multiple Automated Sample Harvester (MASH-II, Microbiological Associates, Bethesda, Md., USA). Filter strips were dried and discs bearing the samples were cut out and placed in scintillation vials containing 0.5% W/V PPO (2,5-diphenyloxazole, KOCH-Light Laboratories Ltd., Colnbrook, Bucks., England) in toluene. Samples were counted in a Packard Liquid Scintillation Spectrometer for one minute each. ^{3}H-thymidine uptake was expressed as the mean number of counts per minute in triplicate sample ± the standard error (cpm ± SE).

2.6.2 *The Cytotoxic Assay*

Tumour cell lines to be used in the cytotoxic assay were harvested from tissue culture flasks into disposable centrifuge tubes. The cells were washed twice in complete medium by centrifugation at 300 g for 10 min. After the final washing, 0.1 ml portions were removed and the cells counted using Trypan blue to indicate the viable cells. Cells were resuspended at a concentration of 2.5×10^{6} viable cells/ml in complete medium; 200 µCi/ml of ^{51}Cr ($Na^{51}CrO_4$ specific activity 194 mCi/mg, Australian Atomic Energy Commission, Commercial Product Unit, N.S.W., Australia) was added to the cell suspension. Tubes were gassed with 10% CO_2, 7% O_2, 83% N_2, sealed and incubated at 37°C for 1 hr. Cells were then washed 3 times by centrifugation at 120 g for 10 min in complete medium, counted and resuspended at a

concentration of 10^5 viable cells/ml in complete medium.

The cell populations assayed for cytotoxicity consisted either of cells pooled from the mixed lymphocyte cultures (set up in 24 well Linbro plastic tissue culture trays) or of cells collected directly from the efferent lymph of sheep immunized with the tumour. In both cases, the cell populations which were used were washed twice, counted and resuspended in complete medium at the required concentrations described in individual experiments. One tenth of a ml of each lymphocyte dilution was added to 4 wells of rounded bottom 96 well trays (Cat. No. 76-011-05, Linbro, USA) containing 0.1 ml target cell suspension. The culture tray was gassed using 10% CO_2, 7% O_2 and 83% N_2, and incubated at 37°C for 4 or 24 hr. Spontaneous ^{51}Cr release was determined by adding 0.1 ml of complete medium to 4 wells containing 0.1 ml of target cell suspension. The maximum amount of ^{51}Cr that could be released was determined by adding 0.1 ml of target cell suspension to centrifuge tubes containing 0.9 ml of distilled water. Tubes containing the cells lysed by water were incubated at 37°C for 4 hr. At the end of this incubation period, the tubes were subjected to 3 cycles of freezing and thawing after which they were centrifuged at 400 g for 10 min. Portions (0.5 ml) of the supernatant of the water lysis controls and 0.1 ml of all other samples were transferred into small tubes and counted in a Packard Gamma Scintillation Spectrometer for one minute each. The percentage cytotoxicity of the samples was derived from the formula:

$$\% \text{ cytotoxicity} = \frac{T - C}{M - C} \times 100$$

where T is ^{51}Cr released in the test sample, C is the spontaneous ^{51}Cr released and M is the maximum ^{51}Cr released.

2.7 ANTIBODY ASSAYS

2.7.1 *Labelling for Immunoglobulins*

All the anti-serum reagents used in this assay were a gift from Dr H.R.P. Miller of the Department of Immunology. The antisera were prepared according to the method described by Nawa *et al.* (1978).

Immunoglobulins on sheep lymphocytes were detected according to the method described by Stanislawski *et al.* (1976). This method is known to stain both intracellular and surface immunoglobulins. In this method lymphocytes were first reacted with rabbit anti-sheep immunoglobulin followed by a reaction with goat anti-rabbit immunoglobulin labelled with horseradish peroxidase. Cytocentrifuge smears of sheep efferent lymphocytes were prepared in Hanks' solution containing 1% bovine serum albumin. Slides were allowed to dry for 15 min at room temperature, after which they were fixed with 4% paraformaldehyde solution for 15 min. They were then washed in 3 changes of PBS for 10 min each and then placed in a moist chamber. Smears were covered with rabbit anti-sheep immunoglobulin (50 µg/ml) and left undisturbed in the humid chamber for 3 hr at room temperature. The slides were then rinsed 3 times for 5 min each in PBS and then returned to the moist chamber. The smears were covered with 50 µl of goat anti-rabbit immunoglobulin labelled with horseradish peroxidase and were left for 45 min at room temperature. The excess immunoglobulin was rinsed off with 3 changes for 5 min each in PBS. After the final wash, smears were stained for 10 min with 3-amino-9-ethylcarbazole/hydrogen peroxide reagent (Graham *et al.*, 1965), rinsed with distilled water and dried. Slides were examined wet using light microscopy

and the number of immunoglobulin-bearing cells were counted in each smear.

2.7.2 Binding Assay

(a) The radioiodination of proteins

All the reagents used for the radioiodination of proteins were precooled, and the entire reaction was performed on ice in low absorption 4 ml plastic tubes. 25 µl of $Na^{125}I$ (Sodium Iodide, S.A. 16.6 mCi/µg. The Radiochemical Centre, Amersham, U.K.) was mixed with 40 µl of rabbit anti-sheep IgG (.25 mg/ml) and 50 µl of Chloramine-T (4 mg/ml, diluted 1/500, British Drug House Laboratory Reagents, Ltd., England) was added, mixed immediately and the reagents were left to react for 60 min. At the end of this period, 50 µl of sodium metabisulphate (6.5 mg/ml, diluted 1/1000, AJAX Chemicals, Sydney, Australia) was added and mixed. This was followed by the addition of 50 µl of 0.1 M KI and the reagents were left on ice for 5 min, after which 0.8 ml of PBS containing 1% bovine serum albumin (BSA) was added. The contents of the small tube were transferred to a small dialysis bag, the tube was rinsed with PBS + 1% BSA and the rinsings transferred to the dialysis bag giving a final volume of 1.25 ml. The bag was dialysed in 3 changes of 60 ml of PBS containing 0.1% sodium azide, for 12 hr each. The sample was then diluted with 3.75 ml of PBS + 1% BSA making the final volume of the solution 5 ml and the final concentration of the IgG, 2 µg/ml. The protein solution was stored at $4^{o}C$.

(b) Quantitation of binding antibodies

A 2 fold serial dilution of a lymph sample in Hanks' solution

containing 1% bovine serum albumin was prepared before the assay was set up. The assay was performed in 96 well microtitre plates (Microtitre, Mic-2000, Cooke Laboratory Products, Alexandria, Virginia, USA) as follows: 50 µl of the serial dilutions of the lymph was dispensed in each well (6 wells for each lymph dilution) and 100 µl of the tumour cell suspension in Hanks' + 1% BSA (10^4 cells) was added to these wells, mixed gently and incubated on ice for 75 min. At the end of this period, 50 µl of Hanks + 1% BSA was added to each well, mixed and centrifuged at 120 g for 7 min at 5°C. The wells were emptied by inverting them once into the sink. The cells were resuspended without adding medium and 150 µl of Hanks' + 1% BSA was added to the cell residue, mixed and centrifuged at 120 g for 7 min at 5°C. This washing process was repeated twice after which 100 µl of ^{125}I-labelled rabbit anti-sheep IgG was added to the cell residue, mixed and left on ice for 75 min. The plates were centrifuged at 120 g for 7 min and the supernatant was removed with a Pasteur pipette. The cells were resuspended and washed 3 times as described above. After the final washing the supernatants from the individual wells were removed, the bases of the plate were cut and placed into small tubes and counted in a Packard Gamma Scintillation Spectrometer for one min each. Controls in the experiment consisted of a row of cells incubated with labelled antiserum and a row of cells incubated in the presence of both normal serum and labelled antiserum. For specificity control, another tumour cell line was included in the assay.

2.7.3 *Cytotoxic Assay*

For the determination of complement-dependent cytotoxic antibodies, all lymph and serum samples were heat inactivated at

56°C for 45 min. Five fold serial dilutions of each sample were prepared using complete medium. Tumour cells to be used in the assay were harvested from tissue culture flasks and labelled with $Na^{51}CrO_4$ as described in Section 2.6.2. The assay was performed in 96 well Linbro tissue culture plates (Linbro, USA) as follows; 50 µl of the complete medium was dispensed into each of the wells, and was followed by the addition of 100 µl of the labelled tumour cell suspension (10^5/ml). A 25 µl volume of the serial dilution of each of the lymph or serum samples was added to 4 wells, followed by the addition of 25 µl of undiluted rabbit complement. The plates were placed in air-tight boxes, gassed with 10% CO_2, 7% O_2 and 83% N_2 and incubated at 37°C for 4 hr. Spontaneous and maximum ^{51}Cr release were determined as described previously in Section 2.6.2. As a control for the non-specific cytotoxic effect of rabbit complement, 25 µl of rabbit complement was added to 4 wells containing a target cell suspension plus complete medium. At the end of the incubation period, 0.05 ml portions of the supernatants from the water lysis controls and 0.1 ml of all other samples were transferred to small tubes and counted in a Packard Gamma Scintillation Spectrometer for one minute each. The percentage cytotoxicity of the samples were determined from the formula:

$$\% \text{ cytotoxicity} = \frac{T - \grave{c}}{M - \grave{c}} \times 100$$

where T is the ^{51}Cr released in the test samples, $\grave{c}$ is the spontaneous ^{51}Cr release in the presence of complement and M is the maximum ^{51}Cr release.

2.8 *IN VIVO* CHALLENGE

2.8.1 Explants

Tumour tissue, to be transferred to recipients was washed 4 times in Hanks' solution containing antibiotics (PSN, 4 times the normal concentration) and cut into fragments measuring approximately 5 × 5 mm. The tumour pieces were implanted by making an incision in the skin with a scalpel, a subcutaneous pouch was then formed, into which the tumour fragment was placed. Antibiotics were added to the wound before the skin was closed using a suture or autoclips. Care was taken during the implantation procedure to avoid any contamination of the wound.

2.8.2 Single cell suspensions

Tumour cells to be used for challenge were harvested from tissue culture flasks as described earlier and washed 3 times in medium without serum. Before the final washing 0.1 ml portions of cell suspensions were removed for counting in Trypan blue and the cells were resuspended at a concentration of 10^7 viable cells/ 0.5 ml of medium without serum.

Before challenge, 0.2 ml of normal saline was infused into the afferent lymphatic cannula followed by the infusion of 0.1 ml of 0.5% T1824 blue dye. The prompt appearance of the dye in the efferent lymph indicated that the lymphatic circuit was intact. Following the appearance of the dye in the efferent lymph, cells were infused slowly into the node followed by an infusion of 0.5 ml of saline to ensure that all the cells were delivered to the node.

2.9 ELECTRON MICROSCOPY

2.9.1 *Reagents*

Cacodylate buffer (0.2M)

4.28 g of Na cacodylate.$3H_2O$ (British Drug Houses, LR, U.K.) was dissolved in 10 ml of distilled water. The pH of the solution was adjusted to 7.2-7.4 with 0.1N HCl. The solution was made up to 100 ml with double distilled water.

Collidine buffer solution (0.2M)

This solution was prepared by adding 2.67 ml of 2,4,6 - Trimethylpyridine (2,4,6-collidine, British Drug Houses, LR, U.K.) to 30 ml of double distilled water. The pH was adjusted to 7.4 with approximately 9 ml of 1N HCl. The solution was made up to 100 ml using double distilled water.

Glutaraldehyde in cacodylate buffer (4%)

This solution was prepared by mixing 10 ml of 10% glutaraldehyde solution (Ladd Research Industries, Burlington, Vermont, U.S.A.) with 15 ml of 0.2M Na cacodylate. The pH of the solution was adjusted to 7.2-7.4 with 0.1N NaOH.

Osmium-collidine fixative

This solution was prepared by mixing 2 parts of 2.0% aqueous solution of OsO_4 (osmium tetroxide, Johnson Matthey Chemicals, London) with one part of 2,4,6-collidine buffer. One drop of 1.0% solution of $CaCl_2$ was added to each 10 ml of the fixative.

Epon 812 resin

The resin was prepared by mixing 2 solutions A & B (1:2). Mixture A contained 62 ml of Epon 812 (Ladd Research Industries,

Burlington, Vermont, U.S.A.) and 100 ml of the hardener DDSA (Dodecenyl Succinic Anhydride, Ladd Research Industries, USA). Mixture B contained 100 ml of Epon 812 (Ladd Research Industries, USA) and 89 ml of NMA (Nadic Methyl Anhydride, Ladd Research Industries, U.S.A.).

Epon and accelerator

This solution was prepared by thoroughly mixing one part of Mixture A with 2 parts of Mixture B for 5 min. Then 0.15 ml of accelerator 964 (Fluka Komponente C, Buchs, Switzerland) was added to each 10 ml of the above mixture. The mixture was stirred for 10 min and then left for 30 min to allow any air bubbles to escape before using the mixture for embedding.

2.9.2 *Processing of Cells*

Cultured cells were harvested with trypsin solution. Cells were washed twice in HBSS after which they were fixed in a pellet form for 4-6 hr at room temperature in 4% cacodylate buffered glutaraldehyde. The cell pellet was rinsed several times in 0.1M Na cacodylate buffer and stored overnight in the refrigerator. The cell pellet was postfixed in osmium-collidine for 2 hr at $4^{o}C$. This was followed by dehydration in graded concentrations of 2 changes of alcohol for 15-20 min each. The pellet was then dehydrated in 2 changes of acetone dried with $CaCl_2$ for 20 min each. The cells were infiltrated with propylene oxide - Epon 812 resin (1:1 mixture) for 60 min. This was followed by infiltration with propylene oxide Epon 812 resin (1:2 mixture) and the samples incubated overnight. Cells were further infiltrated with Epon and accelerator for 4-6 hr at room temperature and then embedded in fresh Epon 812 + accelerator and incubated at $60^{o}C$ for 2-3

days to harden. Thin sections were cut from the embedded material using a diamond knife and an LKB microtome. The sections were stained with uranyl acetate and Millonig's lead stain (Millonig 1961) and examined with a Philips EMU electronmicroscope.

2.10 CELL COUNTS

The concentration of cells in suspensions was determined by one or both of the following methods.

2.10.1 Trypan Blue Exclusion

The Trypan blue dye exclusion test was used to determine viable cell concentrations in all experiments which involved setting up cultures of either tumour cells or lymphocytes. 0.1 ml of cell suspension was mixed with 0.1 ml of 0.125% Trypan blue in PBS, and counted in a haemocytometer to determine the total cell count and the percentage of viable cells. A minimum of 100 cells was counted.

2.10.2 Coulter Counter

This method of cell counting was used for monitoring the response in the efferent lymph collected from stimulated nodes. 0.1 ml of the cell suspension was diluted in 20 ml of PBS and counted using an electronic cell counter (Coulter Model B) with the lower threshold set to exclude red blood cells. Total white cell counts or large cell counts could be obtained by altering the threshold.

Differential cell counts were done on Leishman stained cytocentrifuge smears prepared from efferent lymph from stimulated nodes.

2.11 HISTOLOGY

Tissues were removed and fixed immediately in 4% formaldehyde at room temperature for a few days. Tissues were dehydrated, embedded in paraffin and cut into 7 µm sections. Tissue sections were stained with Mayer's haematoxylin and eosin.

CHAPTER 3

THE ESTABLISHMENT AND CHARACTERIZATION OF THE TUMOUR CELL LINES *IN VITRO* AND IN THE NUDE MOUSE

3.1 INTRODUCTION

The technique of growing neoplastic cells in short-term or established cultures has opened up many new opportunities for studying the neoplastic process. Comparisons can be made between neoplastic cells and their normal counterparts both morphologically and biochemically. From an immunological viewpoint neoplastic cells can be examined for the presence of antigens not found on normal cells. Moreover the cultivated cells may be used *in vitro* as antigens to enable the cellular and humoral immune reactivities of patients from which the tumour cells were derived to be measured.

Similarly, there is an increasing interest in studying the growth of human tumours in immunologically defective animals. Human tumours were first grown in immunologically privileged sites such as the anterior chamber of the eye (Greene 1952), the brain (Greene 1951) and the hamster cheek pouch (Lemon *et al.*, 1952). However, because of the difficulties of using these sites for transplantation studies, attempts were made to grow human tumours in normal animals which had been immunosuppressed by irradiation (Toolan 1951), by cortisone treatment or by both methods (Toolan 1954). More recently, thymectomy (Osoba & Auersperg 1966), and/or treatment with anti-lymphocyte serum (Phillips & Gazet 1967; Cobb 1972) have been used to promote the growth of transplanted tumours. The discovery of the congenitally athymic nude mouse (Flanagan 1966; Pantelouris 1968) provided an excellent opportunity for the use of these animals as a practical system for the study of human tumours in heterologous hosts. Studies have been done on the biology of transplanted tumours, on their

sensitivity to radiotherapy, chemotherapy and immunotherapy as well as other aspects of cancer therapy. Human tumours and their cultivated cell lines have been transplanted serially in the nude mouse (Povlsen and Rygaard 1972; Giovanella *et al.*, 1974) where these tumours have been shown to maintain many of the original characteristics displayed by the primary tumour.

As no reports have been published on the growth of sheep tumours in the nude mouse, attempts were made to establish a number of these tumours as xenografts in this host. Moreover, as a prerequisite to the immunological studies in the present experimental system, it was necessary to attempt to establish cultures of a number of naturally occurring sheep tumours.

The present chapter describes in detail the techniques and procedures used to establish tumour cell lines from clinical cases of epithelial tumours in sheep both *in vitro* and in the nude mouse. Some results are presented on the culture characteristics of these tumour cell lines. These include description of the morphology, ultrastructure and growth rates of the tumour cells *in vitro* and their growth in nude mice. The macroscopic and microscopic morphology of the xenografted tumours was also studied in the nude mouse and compared with the original tumour.

3.2 METHODSOF TISSUE CULTURE

Dulbecco's modified Eagle medium, supplemented with 10% FCS and containing antibiotics (penicillin 100 u/ml, streptomycin 100 µg/ml and neomycin 100 µg/ml), was used for cultivating tumour tissues.

Tissue samples were collected from the sheep into cold

sterile Hanks' solution (HBSS) containing 4 times the normal concentration of antibiotics. Care was taken during biopsy to avoid necrotic or contaminated tissue. Hard tissues were also avoided since it was found after some early experiences that culture of such tissues led mainly to fibroblastic growth. Tissues were transferred to a sterile hood in which they were processed for cultivation.

Because of the high degree of bacterial contamination found within the tumour, it was necessary to wash the tissue fragments extensively in HBSS containing antibiotics (4 times the normal concentration), trim them of any necrotic and contaminated material, and wash them again twice in HBSS and PSN. Tissue pieces were then cut into 1 cm^3 fragments which were kept on ice for 30 min in HBSS and PSN.

The tissue fragments were cut into 2-3 mm cubes using opposed scalpels. Sharp clean cuts were found to be best for allowing subsequent cell growth. Cells failed to migrate from irregular cut surfaces and no growth was observed in these conditions. After the tissue was cut up, fragments were washed 3 times in HBSS and PSN and then processed for tissue culture either by enzymatic digestion using collagenase (Owens *et al.*, 1976) or the pieces were cultivated as explants without any further treatment (Moore *et al.*, 1975).

For cultures prepared by enzymatic digestion, the minced fragments were transferred to 75 cm^2 tissue culture flasks (Falcon plastics, Los Angeles, California, U.S.A.) and dispersed in 15 ml of complete medium containing 1 mg collagenase/ml. The flasks were gassed with 10% CO_2, 7% O_2 and 83% N_2, sealed and incubated at 37^{o}C for 18-24 hr. This incubation was followed by

gently pipetting the tissues back and forth to break up the cell clusters into finer fragments. The flasks were then placed upright for 3-5 min to allow the cell clusters to settle out. The medium was removed with a Pasteur pipette and 15 ml of fresh medium added to each flask. This washing step was repeated twice to remove all the collagenase. The visible cell clusters left after this treatment were redistributed into 25 cm^2 tissue culture flasks with a Pasteur pipette and then fed with 5 ml of the complete medium, gassed with 10% CO_2, 7% O_2 and 83% N_2, sealed and incubated at 37°C.

In cultures prepared by the explant method, 12-15 fragments of tissue were placed directly into 25 cm^2 tissue culture flasks with a Pasteur pipette. A drop of complete medium was then placed on each fragment and the flasks were gassed gently with 10% CO_2, 7% O_2 and 83% N_2, sealed and incubated at 37°C for 1-2 hr. This method of incubation ensured that the fragments adhered to the flasks. Once the fragments adhered, the cultures were fed with 5 ml of complete medium, gassed and incubated at 37°C without disturbing them for 2-3 days. All cultures were fed twice a week by discarding the old medium and replacing it with 5 ml/flask of fresh complete medium. The pH was adjusted carefully at each feeding with 10% CO_2, 7% O_2 and 83% N_2. Care was taken during feeding and gassing of the primary cultures to avoid disturbing the tissue fragments.

3.2.1 *Selection of Epithelial Cells*

Two methods were employed to obtain a pure population of epithelial cells; differential enzymatic treatment and/or mechanical separation.

(a) Differential enzymatic digestion

Cultures were washed twice with trypsin diluent and then subjected to a series of 1-2 min exposures to a trypsin-versene solution (final concentrations of trypsin-versene were 0.25% and 0.1% respectively) followed by washing in trypsin diluent. This procedure resulted in a more rapid detachment of the fibroblastic cells than the epithelial cells. The latter grew in sheets and consequently took a longer time to detach from the flask. By repeating this procedure, most of the fibroblasts were removed and epithelial clusters were left to grow. The epithelial islands were rinsed 3 times with the complete medium, fed, gassed and incubated at 37^{o}C. This procedure sometimes needed to be repeated 2-3 times a week before a pure population of epithelial cells was established.

(b) Mechanical separation

Fibroblasts were identified first using an inverted microscope. These cells were removed mechanically from the culture by scraping the flask with a rubber policeman. The monolayer of epithelial cells was then washed twice to remove all detached cells. Some cultures were separated by enzymatic treatment alone while with others, both enzymatic treatment and mechanical separation methods were used. In those cultures with a high proportion of fibroblasts, mechanical methods were used initially to reduce the numbers of these cells. This was followed by enzymatic treatment.

3.2.2 *Serial Subculture*

For subculture, the monolayer of cells was washed twice

with trypsin diluent and treated with trypsin-versene solution for 10 min. This 10 min treatment resulted in the cells rounding up but they did not become detached completely from the surface of the flask. The trypsin solution was removed with a Pasteur pipette and the flasks tapped to help detach the cells. The cells were then resuspended in complete medium, washed twice and redistributed into new 25 cm^2 or 75 cm^2 tissue culture flasks.

3.2.3 *Morphological Observations*

Tumour cells were cultivated on cover slips in 6-well tissue culture plates (Linbro model FB-6-TC Multidish Disposo-trays) in H-16 and 10% FCS. Morphological studies were done either on live preparations using phase microscopy (Olympus, Tokyo) or on fixed, stained preparations. For electron microscopy, cells were harvested from 25 cm^2 tissue flasks according to the method described previously. The cell pellet was fixed with 4% cacodylate glutaraldehyde fixative, postfixed in osmium-collidine, dehydrated in graded concentrations of alcohol and embedded in Epon.

3.2.4 *Estimation of the Growth Rate and the Doubling Time of the Tumour Cells*

For the determination of growth rates, 1×10^5 cells were inoculated into 25 cm^2 tissue culture flasks in 5 ml of complete medium. The medium was changed every 2 days. At 24 hr intervals, 3 cultures were harvested using the method described earlier and the cells were counted in a haemocytometer. The doubling time of the cell population, (the time required for the cell population to double in number), was determined from the growth curves (Huh *et al.*, 1977). Saturation density was taken as the

value where 3 successive harvests showed no increase in cell number (Owens *et al.*, 1976).

3.2.5 *Capacity of Cultivated Cells to Produce Tumours in Nude Mice*

The ability of cultivated cells to grow in immunosuppressed hosts was used as a measure of their malignancy (Sincovics *et al.*, 1978). Cultivated tumour cells were injected intradermally, subcutaneously or intraperitoneally into nude mice and their subsequent growth was observed. Explants were also taken directly from tumour-bearing sheep and transplanted into nude mice (see Section 3.3.6 in this chapter).

3.3 RESULTS

3.3.1 *The Establishment of Tumour Cell Lines*

Fifty different tumours were biopsied and used for establishment of cultivated cell lines. From these 50 tumours, 17 cell lines were established; a success rate of 34%. Table 3.1 shows detailed data of the established cell lines in regard to the site of tumour, the methods used for the initial establishment of the cultures and the methods used for subsequent separation of epithelial cells from fibroblasts.

In the early attempts to establish cultures, both enzymatic digestion and explant techniques were used. Only 3 cultures were established from 25 tumours, using the enzymatic treatment (12%) compared to 7 cultures established by the explant method (28%). Therefore, for subsequent attempts only the explant technique was used and the success rate improved to 40% (Table 3.2).

The failure to establish cultures by enzymatic treatment

Table 3.1 *Methods of establishment and isolation in culture of ovine squamous cell carcinomata derived from different biopsy sites*

Cell line number	Site of biopsy	Method of establishment	*Method of isolation
F 6	nose	explant	enzymatic treatment
F 18	"	"	"
F 40	"	"	"
F 41	"	"	enzymatic & mechanical
F 43	"	"	"
F 47	"	"	enzymatic treatment
F 59	"	"	mechanical separation
F 61	"	"	"
F 31	ear	#enzymatic	"
F 34	"	explant	enzymatic treatment
F 35	"	"	"
F 39	"	#enzymatic	mechanical separation
F 51	"	explant	enzymatic treatment
F 54	"	"	"
F 57	"	"	"
F 58	"	"	enzymatic & mechanical
F 24	vulva	#enzymatic	mechanical separation

* Methods used for separating epithelial cells from fibroblasts in the initial culture.

\# All three cultures prepared by enzymatic treatment were also grown successfully by the explant method.

was not due to any difficulties encountered in the establishment of the initial culture but to problems arising in the subsequent purification of the epithelial cells. This may have been due to the fact that in explant culture, cells migrate from the explant outwards to form sheets of cells, whereas in cultures prepared by the enzymatic digestion, cells grow in small colonies completely surrounded by colonies of fibroblasts. Epithelial cells growing in the latter situation seem to be more sensitive to trypsin treatment, and they detach at approximately the same time as the fibroblasts. For this reason mechanical separation was used to separate epithelial cells from fibroblasts in these cultures.

A correlation between the site of biopsy and the success rate in establishing cultures was observed in these experiments. The total success rate in cultivating these tumours was 34%. Table 3.3 shows that out of 14 attempts to establish cultures from tumours on the nose, 8 were successful (57%). With tumours from the ear, 8 cultures were established out of 22 attempts (36%). Only one culture was established from tumours derived from the vulva out of 13 attempts (7.6%). The reasons for the difficulties found in establishing cultures from tumours growing on the vulva is not known. However from my own experience, difficulties were mainly related to the initial establishment of the epithelial growth rather than to any subsequent difficulties in the method of isolation. Two other cell lines were established from this region, however they were lost (degenerated) on subsequent subcultivation (2nd and 3rd).

3.3.2 *The Morphology of Established Cultures*

In enzymatically prepared cultures, islands of epithelial

Table 3.2 *The success rate for the cultivation of ovine squamous cell carcinomata using either the explant or enzymatic digestion techniques.*

Method of culture	Number of attempts	Number of established cultures
Enzymatic	25	3 (12)*
Explant	25	#7 (28)
Explant	25	10 (40)

Success rate by explant 14/50 (28%)

* The number in parenthesis represents the percentage success rate.

\# These cultures include the 3 cultures established by both methods.

Table 3.3 *The relationship between the site of the tumour and the success of cultures.*

Site of tumour	*Number of established cultures	% success rate
eye	0/1	0.0
vulva	1/13	7.6
ear	8/22	36.4
nose	8/14	57.0
total	17/50	34.0

*Number of established cultures/number of attempts.

cells were seen as early as 24-48 hr after the cultures were initiated (Fig. 3.1). In explant cultures, the outgrowths of epithelial cells from the tissue fragments were observed 72-96 hr after the cultures were set up (Fig. 3.2). Of the 17 cell lines established, two distinctive morphological types of cells were observed. Fig. 3.3 shows one of these cell types. Epithelial cells were small in size, densely packed and had a regular cobblestone appearance. The nuclei of these cells were large relative to the cytoplasm. Cells were frequently observed in mitosis in these cultures. When the cells became confluent they usually continued to grow, piling up on each other. This suggested that these cells were not subjected to any contact inhibition (Fig. 3.4).

The other morphologic cell type observed in the established cell lines is shown in Fig. 3.5a. Cells in these cultures were irregular in shape and size, and multinucleated giant cells were frequently seen (Fig. 3.5b). The nuclei of these cells were small in comparison to the cytoplasm which was highly vacuolated. Cell division was also frequent in these cultures and abnormal mitotic figures were regularly seen. Cellular degeneration occurred in some of these cultures when they reached a confluent state. Variations were observed in the amount of vacuolation of the cytoplasm and in the number of giant cells between the different established cultures.

Cells were tested for the presence of keratin to authenticate their epithelial character. Of 8 cell lines tested, 6 stained positively for keratin (Fig. 3.6) and these cultures remained positive throughout each subculture.

3.3.3 *Ultrastructural Characteristics of Cultivated Tumour Cells*

Five cultivated tumour cell lines were examined in the electron microscope after the cells were removed from the culture flasks by trypsinization. Most of the cells were disassociated by this treatment but some small aggregates remained intact as fragments of the original monolayers.

The cultivated cells were typical epithelial cells, differentiated to varying degrees. Some cells were epidermal keratinocytes, many with the characteristics of granular cells, while others had the characteristics of cells from the basal or germinative layer. There were no fibroblasts, Merkel cells or Langerhans cells identifiable in any of the established cell lines.

(a) Keratinocytes

Typical examples of these cells are shown in Figures 3.7, 3.8 and 3.9. The number of keratohyalin bodies and the amount of keratin present in these cells varied. The cytoplasm of some cells was filled with tonofibrils and associated keratohyalin (Fig. 3.10, 3.11) while others contained only a few scattered keratohyalin bodies. The keratin fibrils and keratohyalin bodies were located principally in and around the Golgi area of the cells and in a thin marginal area surrounding the nucleus (Fig. 3.7, 3.8). In the cell undergoing mitosis the keratin formed a circumferential ring near to the cell membrane following the dissolution of the nuclear membrane (Fig. 3.12). Many of these cells had a highly vacuolated cytoplasm.

In the more differentiated cells there were large amounts of keratin and tonofibrils; no cells in the cultures developed

to the stage of cornification. There was a variety of inclusions in the keratinocytes including lamellar granules, melanin and lipid granules as well as lysosomes (Fig. 3.10, 3.11, 3.13a, 3.13b). Almost all these cells had large prominent nucleoli with an open reticulated structure comprised of RNA granules. Their cytoplasm contained numerous polyribosomes, arranged in clusters and spirals (Fig. 3.14). There was no visible glycogen in the cytoplasm of these cells.

(b) Basal Cells and Spinous Cells

There were large numbers of cells in the cultures that resembled basal cells and cells with the characteristics of more differentiated spinous cells. The cytoplasm of the basal cells was filled with glycogen granules arranged singly or in clusters (Fig. 3.15). There were very few or no keratohyalin bodies in these cells and the tonofibrils were scattered through the cytoplasm and not aggregated into distinct bundles (Fig. 3.16a). Basal cells still associated with one another were joined by desmosomes. The desmosomal boundaries were thickened and more electron dense than the adjacent plasma membrane (Fig. 3.16b) but there was no orientation of the tonofibrils to the desmosomal attachments. The cytoplasm of the basal cells contained many polysomes but no rough endoplasmic reticulum. Basal cells were seen in mitosis in the cultures.

The cells which resembled spinous cells had more tonofibrils than the basal cells and also more keratohyalin in their cytoplam (Fig. 3.17). These cells were proliferating rapidly in culture and the mitotic figures usually showed dispersed profiles of keratohyalin arranged peripherally in the cytoplasm. Some glycogen

was often present in their cytoplasm. The cytoplasm of the basal and spinous cells was significantly less electron-dense than that of the more differentiated keratinocytes.

There were some cells in the cultures that had a few rough endoplasmic reticular profiles in their cytoplasm and cisternae filled with secretory products (Fig. 3.18a, 3.18b).

There were also a variety of other cells with highly vacuolated cytoplasm and a variety of inclusion bodies. These cells did not have any keratin or tonofibrils in their cytoplasm and their designation was uncertain.

3.3.4 *Growth Rate and Doubling-Time of Cultivated Tumour Cells*

In the primary cultures, epithelial cells had a very slow growth rate and took almost one month to reach confluency in 25 cm^2 flasks. However with subsequent subculture, epithelial cells grew at much faster rates and usually reached confluency within a week.

Growth curves were established for different cell lines after the same number of subcultures. In general two different types of growth curves were obtained. One pattern of growth had a lag phase following subculture. This was followed by an increase in cell numbers until saturation density was reached. After this the cell numbers declined (Fig. 3.19a). A second pattern of growth was observed in which there was no lag phase. Cell numbers began increasing within 24 hr of subculture until they stabilized (Fig. 3.19b). In the pattern of growth in which there was a lag phase, the different cell lines differed in the length

of the lag period as well as in the final cell density. In some, the final density of cells correlated with confluency, while in others, cells ceased to grow even though they had not become a confluent monolayer. In the growth pattern with a lag phase, there was a slower growth rate and saturation density was reached 48-72 hr later than with cells that showed no lag period in their growth.

An increase in the growth rate was observed following subculture *in vitro*. Fig. 3.20 shows the growth rates determined for the cell line F43 at different subculture levels. At the 6th subculture, the doubling-time was 88 hr, whereas at the 10th and 20th subculture, the doubling-time was 54.4 and 26.6 hr respectively.

3.3.5 *Xenotransplantation of Cultivated Tumour Cells*

The ability of cultivated tumour cell lines to grow in nude mice was assessed by xenogeneic transfer. Tumour cells were injected either intradermally, intraperitoneally or subcutaneously into nude mice and the subsequent growth of the tumour was followed. In all 3 cell lines tested, cells established growth following intradermal injection whereas none grew following intraperitoneal or subcutaneous injections (Table 3.4). Further cell lines that were tested were therefore injected intradermally. Table 3.5 shows the results of injection of 8 different cultivated tumour cell lines into nude mice. Four out of the 8 cell lines tested gave rise to tumours. With some tumours, small nodules appeared at the innoculation site 2-3 days following injection. These nodules enlarged over the next 2 months reaching a size of between 15 × 15 mm and 25 × 25 mm by approximately 60 days

Table 3.4 *The effect of route of injection on the growth of inocula of tumour cells in nude mice.*

Cell line number	Number of subcultures	Route of injection	Tumour size (mm)	Days observed
F6	4	Intradermal	11 x 8	52
		*Intraperitoneal	-	89
		Subcutaneous	-	120
F18	6	I.D.	18 x 17	35
		I.P.	-	135
		S.C.	-	85
F43	4	I.D.	8 x 5	26
		I.P.	-	94
		S.C.	-	111

- No growth

Number of cells injected was 10^6.

* animals receiving ip tumour cells were sacrificed at the end of the observation period, dissected and examined macroscopically for the presence of tumours.

Table 3.5 *Tumour production in nude mice following intradermal injection of cultivated tumour cell lines*

Cell line number	Number of subcultures	Numbers of cell injected	Tumour size (mm)	Days observed
F6	6	1×10^6	15 x 15	60
F18	8	1×10^6	25 x 25	55
F43	6	1×10^6	17 x 15	49
F40	9	*1×10^7	-	123
F35	5	1×10^7	16 x 18	29
F51	5	1×10^7	-	92
F58	7	1×10^7	-	139
F24	3	1×10^7	-	150

* Mice were injected with 1×10^7 cultivated tumour cells because in earlier attempts, when mice were injected with 1×10^6 tumour cells, no growth was observed over a period of 3-5 months.

(F6, F18 and F43). The other tumour cell line injected into the nude mice (F35) had a latency period of 7-10 days before it appeared at the inoculation site as a small nodule. After the nodule appeared growth was rapid reaching an average size of 16 × 18 mm within the next 30 days. No regression was observed in any of the cell lines which showed initial growth.

3.3.6 *Xenotransplantation of Tumour Explants*

All transplantation procedures were done under sterile conditions. Tissue samples were collected in sterile cold HBSS containing antibiotics (PSN). Tissues were taken from healthy well-vascularized areas at the periphery of the tumour; care was taken to avoid necrotic areas. Tissue fragments were divided into 2 portions; one portion was fixed for histological examination and the other was used for implantation.

A 6-8 mm incision was made in the lateral abdominal wall of the mouse and a subcutaneous pouch formed by blunt dissection. A piece of tumour tissue measuring approximately 3 × 3 mm was inserted into the pouch and positioned on the lateral thoracic wall, at least 8 mm away from the incision. The incision was then closed using autoclips (Clay Adams, Parisppany, N.J.). After tumour implantation, mice were observed daily and the growth of the tumour was measured twice a week with calipers. Tumour volume was estimated from the formula $(\Pi/6)d^3$ where d is the mean diameter of the tumour (Selby *et al.*, 1980).

When the tumours had reached a sufficient size or had killed the host, they were dissected free and examined. The autopsy was done by dissecting the skin over the tumour site to allow for its inspection. Lymph nodes were examined for any

enlargement, and the thorax and abdomen were opened and internal organs examined. For histological studies, tissues were taken from the tumour at its first transplantation. Randomly chosen tumours which had been grown serially were sampled and prepared for microscopic examination. Organs which showed macroscopic changes and enlarged lymph nodes were also prepared for histology.

Twenty five different tumours from various sites on sheep were implanted subcutaneously into nude mice (Table 3.6). Of these tumours 10 tumour lines were established and subjected to serial transplantation. Of the remaining 15 tumours transplanted, 7 failed to establish any growth, while 5 others grew initially and formed small nodules before they eventually regressed. The remaining 3 tumours grew progressively for 2-3 months following their initial implantation but they were lost in subsequent passages.

Of the primary tumour grafts, 13 (52%) were successful in the first transplantation, 10 (40%) of these were subsequently transplanted serially. A possible correlation between the site of the original tumour growth and success in establishing growth in nude mice was observed in this study. Two xenografts were established from tumours growing on the vulva out of 7 attempts (28%). Three out of 9 tumours transplanted were established from tumours derived from the ear (33%), and 5 out of 9 were established from tumours on the nose (56%). Thus tumours from the nose had the highest rate of establishment when transplanted into nude mice followed by those on the ear and vulva. The rate of acceptance of tumour grafts in the first transplantation was variable and in some cases it was extremely low. However, the rate of acceptance of established tumours in serial transplantation varied between 80-100% (Table 3.7).

Table 3.6 *Details of the history of the primary transplantations of sheep tumours into nude mice*

Sheep number	Tumour site	Growth observed	#Numbers accepted	Passage number
F 4	Vulva	-	0/6	-
F 7	"	-	0/6	-
*F 19	"	+	1/4	-
F 21	"	+	6/6	8
*F 23	"	+	2/6	-
F 24	"	+	1/6	7
*F 38	"	+	6/6	-
*F 5	ear	+	1/6	-
F 14	"	-	0/6	-
†F 31	"	+	2/6	-
F 33	"	+	3/6	6
F 34	"	-	0/6	-
F 35	"	+	2/6	7
†F 36	"	+	2/6	-
F 37	"	+	1/6	9
F 44	"	-	0/5	-
F 6	nose	+	1/14	15
F 18	"	+	3/6	20
F 29	"	-	0/6	-
F 40	"	+	2/6	6
F 41	"	+	1/6	11
F 42	"	-	0/6	-
*F 43	"	+	1/6	-
†F 46	"	+	1/4	-
F 47	"	+	1/4	5

number of animals accepting the tumour graft/number of ainimals inoculated.

* tumours remained as small nodules throughout the observation period (4-5 months)

† tumours grew initially but disappeared on subsequent passages

+ yes, - no.

Table 3.7 *Acceptance rates of selected tumours following serial passage*

Tumour number	Passage number	*Acceptance rate	Percentage
F18	1	3/6	50
	2	5/6	83
	7	6/6	100
F6	1	1/14	7
	2	12/12	100
	5	6/6	100
F35	1	2/6	33
	2	8/9	89
	7	7/8	88

* Number of animals accepting tumour graft/number of animals inoculated.

3.3.7 *Tumour Morphology*

The pattern of growth of the different tumours established in the nude mice by transplantation of explants was very similar to the growth of the primary tumour in the original host. The morphologic appearance was uniform and consistent with each subsequent transplantation in the nude mouse. Basically two distinctive morphologic types of tumours were observed in nude mice.

(a) Expansively growing tumours

Tumours of this type began to increase in size usually between 10-14 days after they were transplanted. In early passages, growth was slow until the tumour became established (approx. vol. 1.5 cm^3); growth then increased rapidly. The tumour attained an approximate volume of 12 cm^3 within 2-3 months of transplantation. As shown in Fig. 3.22, the tumours had smooth surfaces, were well circumscribed and encapsulated. These tumours remained movable and did not become attached to either the skin or the chest wall. The tumours invariably had a sterile necrotic centre (Fig. 3.24c) which was revealed when the tumour was cut. The extent of necrosis was proportional to the tumour volume. This type of growth was identical to that seen in the host sheep (Fig. 3.21).

Histologically the transplanted tumours were differentiated squamous cell carcinomata with some areas of keratinization (Fig. 3.24a). The boundary between the normal tissue and the tumour was well demarcated by a thick layer of connective tissue elements (Fig. 3.24b). This histological appearance was retained with tumours that had been serially transplanted and resembled closely the histological appearance of the primary tumour (Fig. 3.23). In one case (F18), the amount of stromal material within

the tumour decreased with subsequent passages. Lymph nodes and other organs showed no tumour metastases in the nude mouse.

(b) Invasively growing tumours

Tumours of this type grew very slowly in the first 2 months after the primary transplantation. Growth was more obvious during the 60-90 days following transplantation and the tumours reached a volume of 2-3 cm^3 by this time. As shown in Fig. 3.26 the tumours were highly vascular with rough surfaces. They were not encapsulated but extended into the adjacent tissues. These tumours sent out finger-like projections of malignant tissue which formed adhesions with the overlying skin and the underlying body wall (Fig. 3.27). Ulcerations occurred through the skin with this tumour, but there was no internal necrosis. This invasive type of growth corresponded closely to the way these particular tumours grew in their primary hosts. For example invasion of the upper lip and destruction of the cartilageneous elements in the nasal septum was observed in sheep carrying these tumours (Fig. 3.25).

The histological appearance of some of these tumours was characterized by the presence of spindle-shaped tumour cells separated by large amounts of stromal elements. Few keratinized areas were observed scattered throughout the tumour (Fig. 3.29). These tumours had a similar histology to the primary tumour (Fig. 3.28), and their histological appearance remained unchanged on subsequent passages. The tumours were locally invasive and infiltrated the adjacent tissues. In a cross section through the muscle layer overlying the thoracic wall, clusters of tumour cells could be seen surrounding individual muscle cells (Fig. 3.30).

In one case a secondary growth of the tumour was found in the lung (Fig. 3.31). Tumour tissue had already penetrated the body wall by direct extension and was also located on the inside of the thorax and extended over the surface of the lung. The histologic appearance just described was representative of some infiltrative tumours. Other infiltrative tumours had a histologic appearance similar to that described for tumours which had an expansive growth. No correlation between the histologic appearance of the tumour and its growth pattern was observed.

3.3.8 Growth Rates

Growth curves (expressed as volume) were plotted for various tumours after varying numbers of transfers. Differences were observed in the growth rates of the tumours with subsequent passages. Fig. 3.32 shows the growth rates of 2 representative tumours (F6 and F18) after the first, 7th and 9th passage. Cell line F6, which is an example of an invasive tumour had a latency period of 2 months before any growth of the tumour was observed at its primary transfer. After this period the tumour increased in size and reached a volume of 2 cm^3 by 90 days following transplantation. When subsequently transplanted, the latency period was shorter (20 days) and the tumour reached a volume of 2 cm^3 in 50-60 days. For the other tumour, F18, (expansive growth), the latency period during which no growth was observed was between 10-15 days at the primary transfer; after this time there was a dramatic increase in the size of the tumour which reached a volume of approximately 5 cm^3 by 55 days after it was transplanted. Faster growth rates were observed with subsequent passages of the tumour and it reached a volume of 1·2 cm^3 on day 65

and day 55 on the 7th and 9th passages respectively.

3.4 DISCUSSION

These experiments have demonstrated that pure populations of cells derived from ovine squamous cell carcinomata can be grown successfully *in vitro*. A number of factors were found to influence the success in establishing the cell cultures. These included the initial method used for obtaining the cells, and the subsequent method used for separating the epithelial cells from contaminating fibroblasts. The site of the tumour appeared also to influence the success of the culture. A high success rate was obtained in tumours derived from the nose, success was less with tumours growing on the ear and least with tumours from the vulva. The reason for this association between the site of the tumour and the success rate in establishing cultures is not known, but it does not seem to be related to the histological appearance of the tumour. However, it may be related to the malignancy of the tumour, since most tumours growing on the nose tended to be invasive, infiltrating the nasal cartilage and the adjacent tissues of the upper lip.

Comparative studies were done on the relationship between the location of the tumour, its growth pattern (invasive or expansive) and the growth rate and morphologic appearance of the established cell lines *in vitro*. No association was found between the tumour site and the morphologic appearance of the tumour or between the growth pattern of the tumour and its morphologic appearance. However there seemed to be an association between the growth pattern of the tumour in the sheep and the

growth rates of the cultivated cells *in vitro*. It was found that cells from all invasive tumours, except two (F6 and F43), grew relatively slowly *in vitro* when compared with other cell lines after the same number of subcultures.

An increase in the growth rate of the established cell lines was observed following *in vitro* subculture. This may have been due either to the adaptation of the cells to grow *in vitro*, or, because the cell population was heterogeneous, tissue culture may have resulted in selection of fast growing cells which subsequently predominated in the culture. These results agree with data reported by Huang *et al.* (1980) who observed that a tumour cell line established from the nasopharynx showed an increase in its growth rate following *in vitro* subculture. However the growth rate of this culture stabilized at the 25th subculture.

Some cultivated cell lines failed to grow when inoculated into nude mice. This may be due to the fact that growth is dependent on the preservation of the architecture of the tumour parenchyma and stroma (Povlsen and Rygaard 1976). However other reasons such as an insufficient inoculum size or insufficient time elapsing between the time of tumour inoculation and the death of the nude mouse may explain the failure of growth.

Successful transplantion of sheep tumours from different sites into nude mice was also established in 10 out of 25 attempts. As with *in vitro* culture, there was a correlation between the site where the tumour was located and its acceptance as a xenograft. Tumours derived from the nose were most readily accepted followed by those derived from the ear and vulva respectively.

Three other tumours were transplanted into nude mice and their growth observed. While these tumours grew on first passage,

they failed to grow when transplanted to secondary hosts. This may have been due to the fact that these particular tumours showed massive central necrosis to the extent that only a thin outer rim of tumour tissue was available for transplantation. This lack of a sufficiently large inoculum could have been the reason behind the failure of these tumours to grow when passaged subsequently.

The other five tumours which remained the same size throughout the observation period (4-5 months) may have required a longer period of time to grow (Povlsen and Rygaard, 1976). The life span of the nude mouse did not permit these studies to be followed except by keeping the mice in germ-free conditions. The inability of these tumours to grow could have also been due to other factors such as a lack of vascularization or to the depletion of an essential factor for tumour growth which is present in sheep but not in nude mice.

The reason for the complete failure of the remaining 7 tumours to grow is unknown. One possible explanation may be that the failure of these tumours to grow is due to their immune rejection by the nude mouse. Although the nude mouse lacks a thymus, it is not completely incompetent immunologically. It has been shown that nude mice have intact humoral immune mechanisms (Koene *et al.*, 1974, Adelman *et al.*, 1980) and they have an enhanced activated macrophage response to bacterial infection (Reid *et al.*, 1978) as well as an enhanced NK cell response when compared to conventional mice (Holden *et al.*, 1978). The increased NK cell activity may account for the low incidence of spontaneous tumours in the nude mouse (Outzen *et al.*, 1975). Since NK cell activity is highest at 5-10 weeks of age and since most of the

mice used in my experiments fell within this age group (6-8 weeks old), it is possible that the lack of growth of some of these tumours was due to rejection by NK cells.

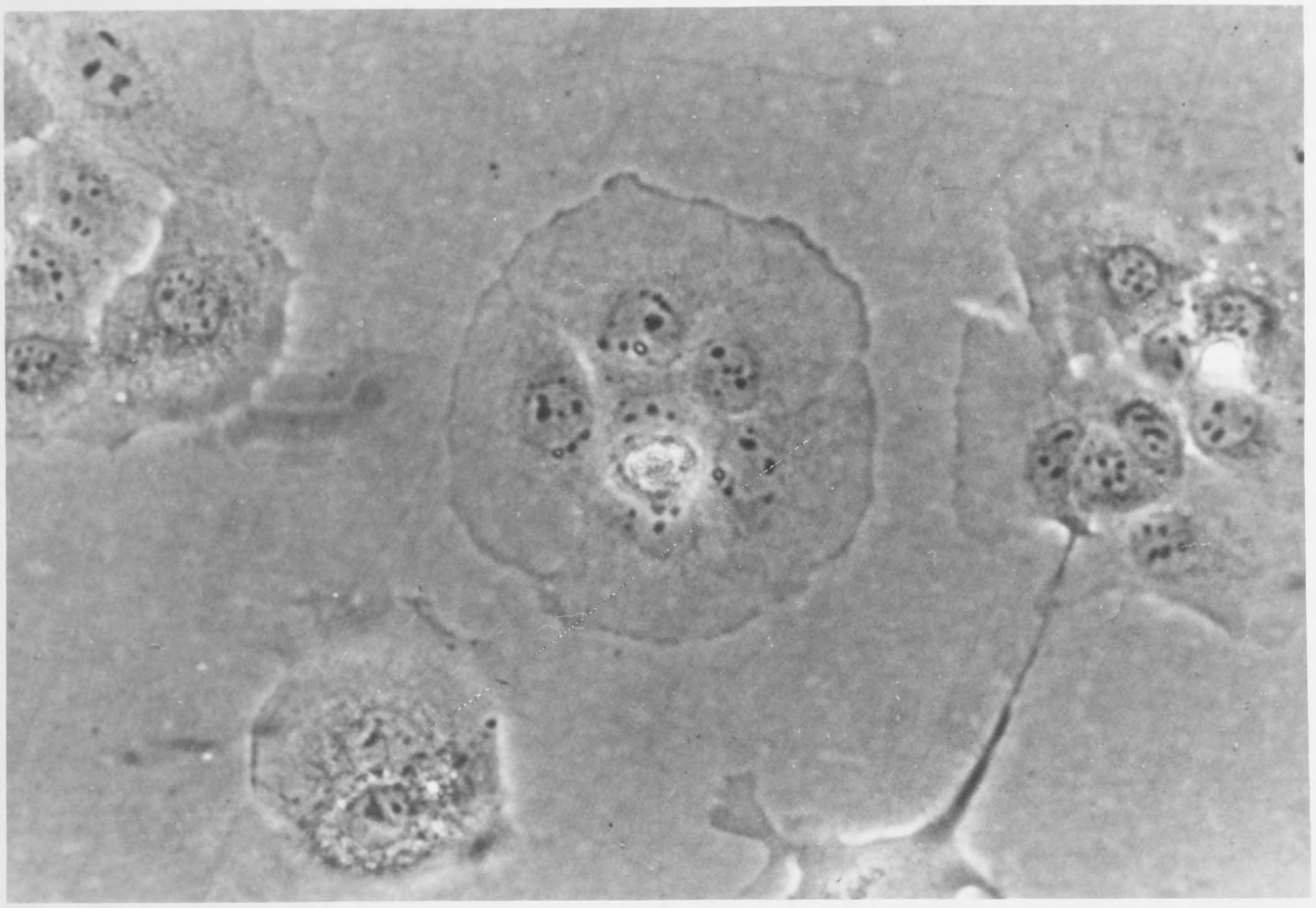
The pattern of growth of the tumours in the nude mice was similar to that observed in sheep. Thus the F18 tumour was a highly vascular tumour in the sheep where it grew expansively and showed no tendency to infiltrate or metastasize. F6 was a highly infiltrative tumour, invading the surrounding tissues of the upper lip and the nose cartilage. This same invasive pattern was observed in the nude mice to which the tumour was transplanted. This finding is not in accord with the findings of Povlsen and Rygaard (1971). They reported a change in the growth pattern of human carcinomata of the rectum and colon once these tumours were transplanted into nude mice. Although the rectal and colonic tumours had an invasive growth in the primary host, they grew as local well-circumscribed tumours in nude mice. The reason behind this difference is unknown, however it may be related either to the site from which the primary tumour was obtained or to the sex of the nude mouse. Reid *et al.* (1978) have described a prostatic carcinoma which was found to be locally invasive to the muscles when transplanted into females. However, this prostatic carcinoma grew as a circumscribed encapsulated tumour when it was transplanted into male mice.

The histological appearance of the skin tumours in mice resembled that in the primary host, and did not change through subsequent passages except in the case of one tumour (F18) where a decrease in the amount of stromal elements within the tumour was observed on serial passage. This may account for the increase in the acceptance rate of this tumour as well as the increase in

its growth rate observed in its later passages. Loss or change in the stromal content of some transplanted human tumours have also been reported by other investigators (Povlsen and Rygaard 1971, Selby *et al.*, 1980). However this loss was attributed to the gradual replacement of the fibroblastic elements within the primary tumour by fibroblasts of mouse origin (Selby *et al.*, 1980)

Figure 3.1 Epithelial islands growing from an enzymatically prepared culture, 48 hr after culture initiation.

Figure 3.2 Epithelial cells growing from a tissue fragment in explant culture, 72 hr after culture initiation.



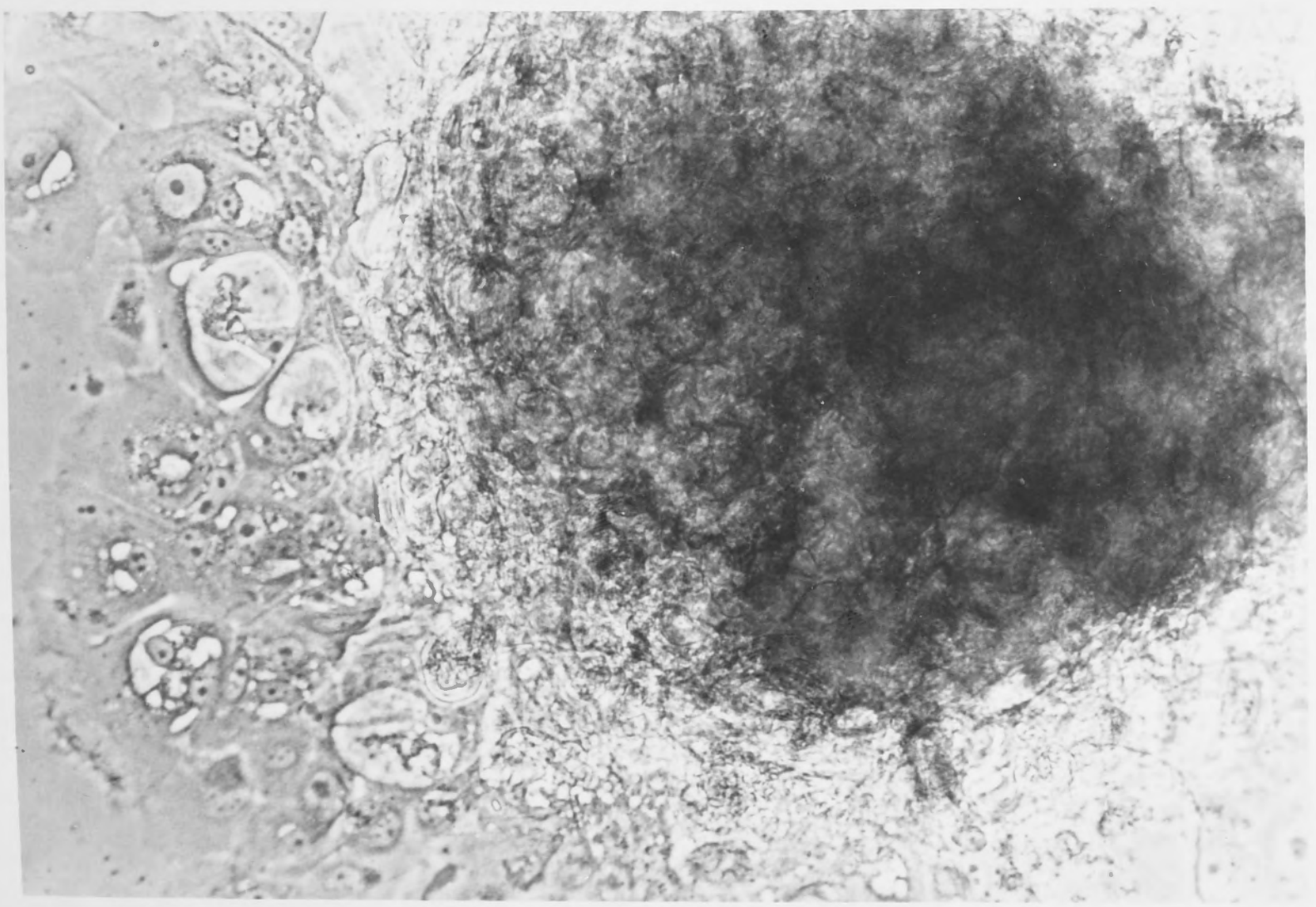
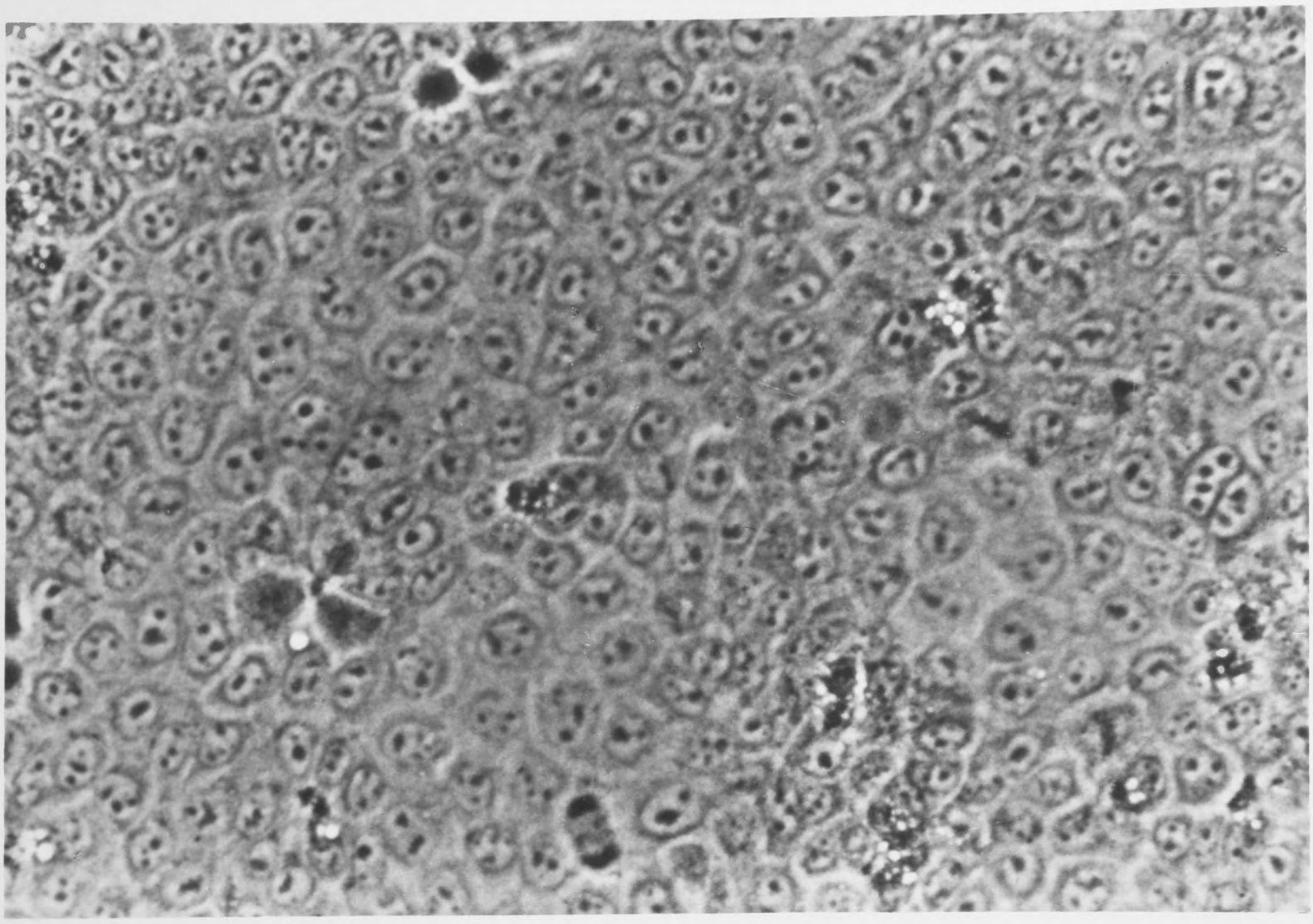


Figure 3.3 Epithelial cells of culture F6 (5 days after the 4th subculture) showing a regular cobblestone appearance with large nuclei; some cells are seen in mitosis.

Figure 3.4 The morphologic appearance of the cell line shown in Fig. 3.5, taken 9 days after subculture. Note loss of contact inhibition with subsequent piling up of cells.



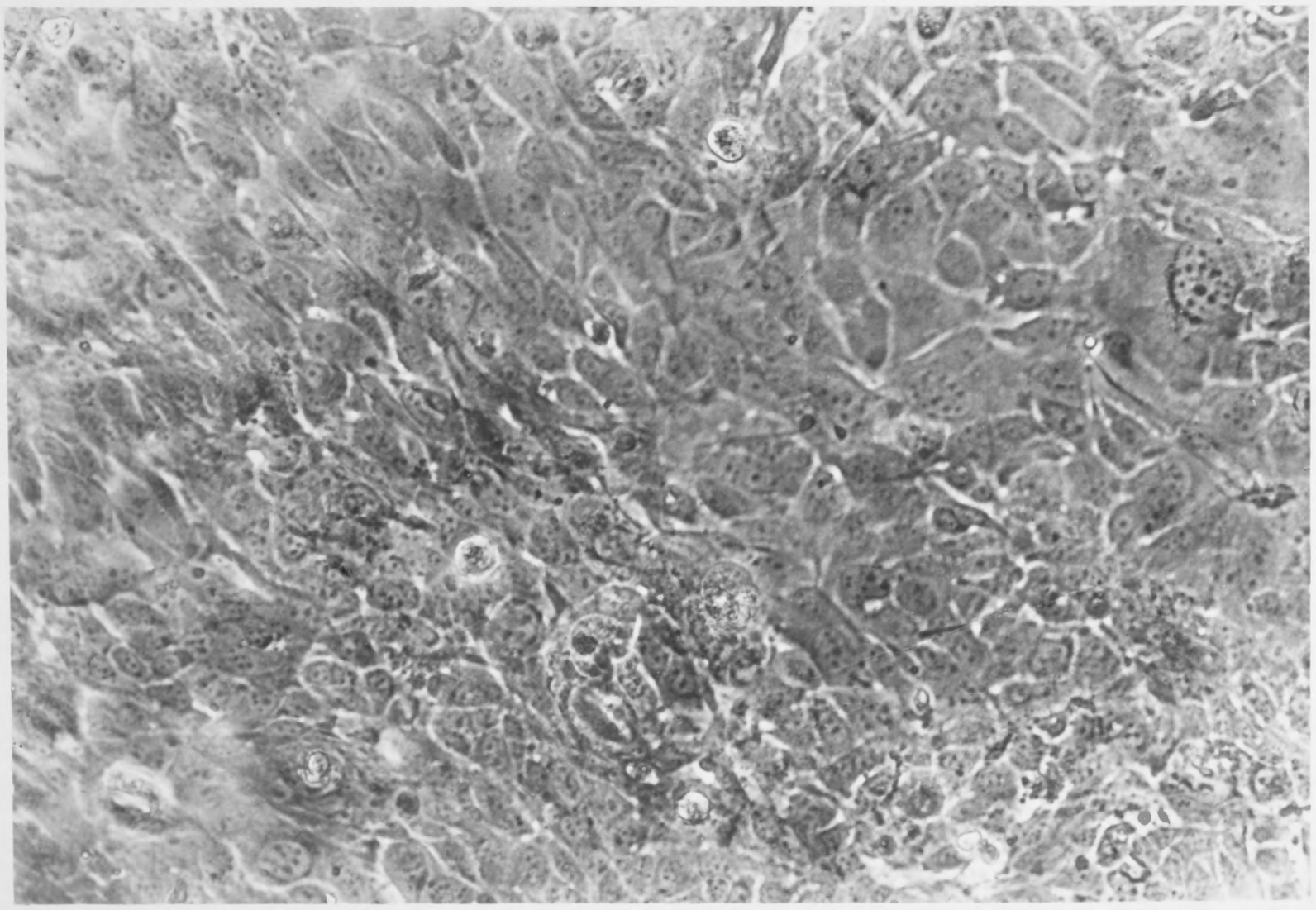
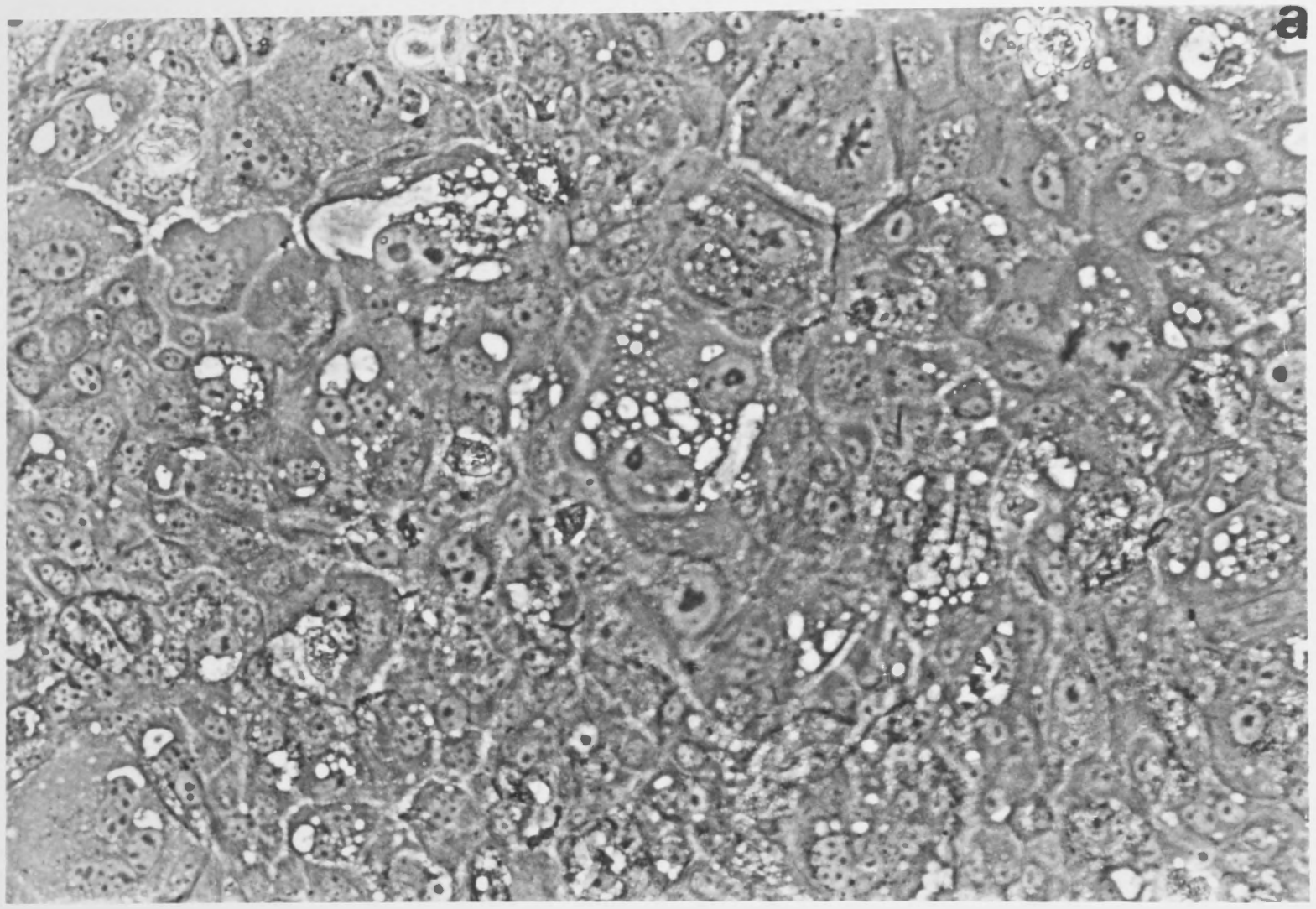


Figure 3.5 The morphologic appearance of Culture F18 5 days after the 4th subculture:

(a) Irregular growth pattern of cells in the culture, the cytoplasm of each cell is large compared to the nucleus and it is highly vacuolated.

(b) Multinucleated giant cells are frequently seen in the culture.


a

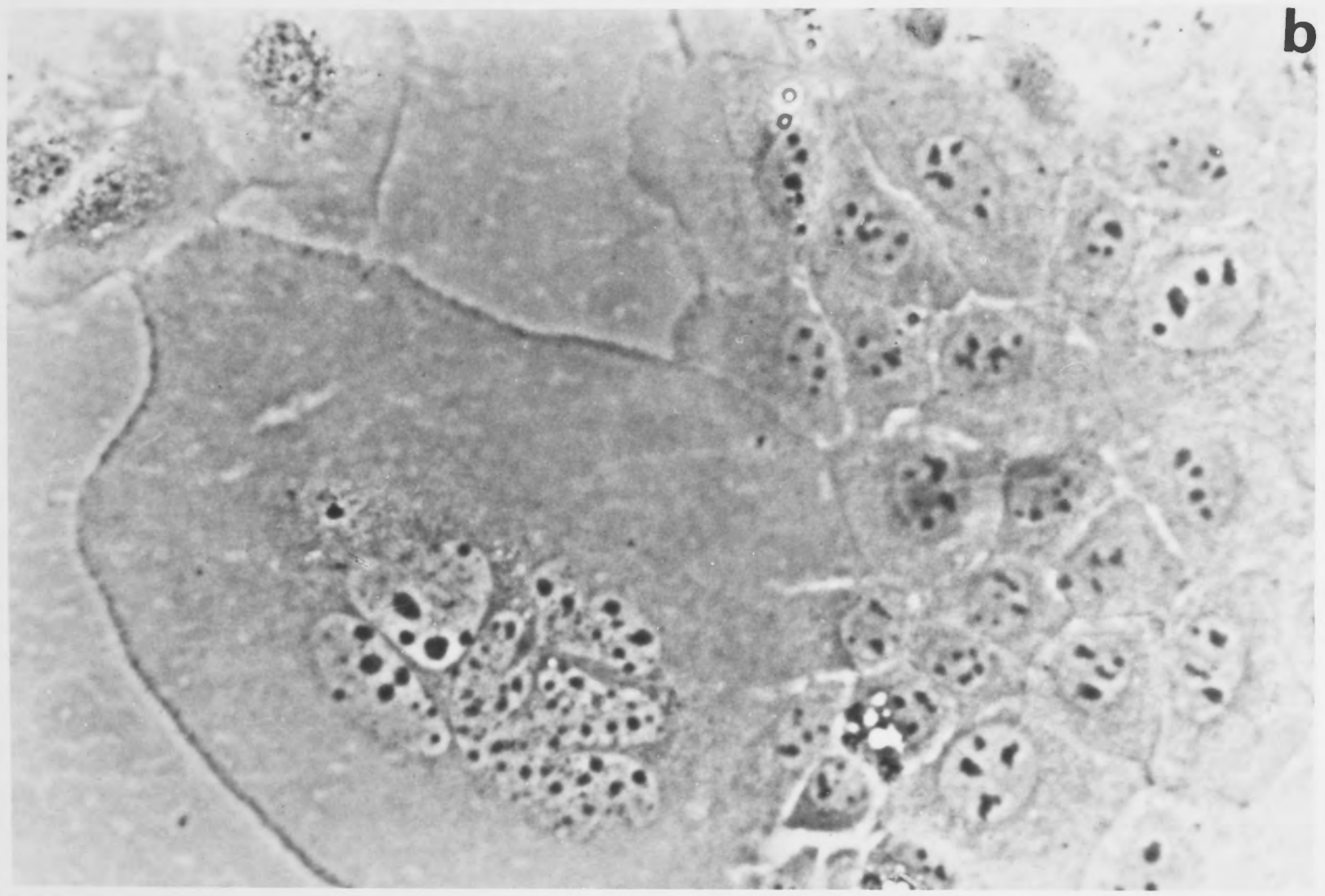
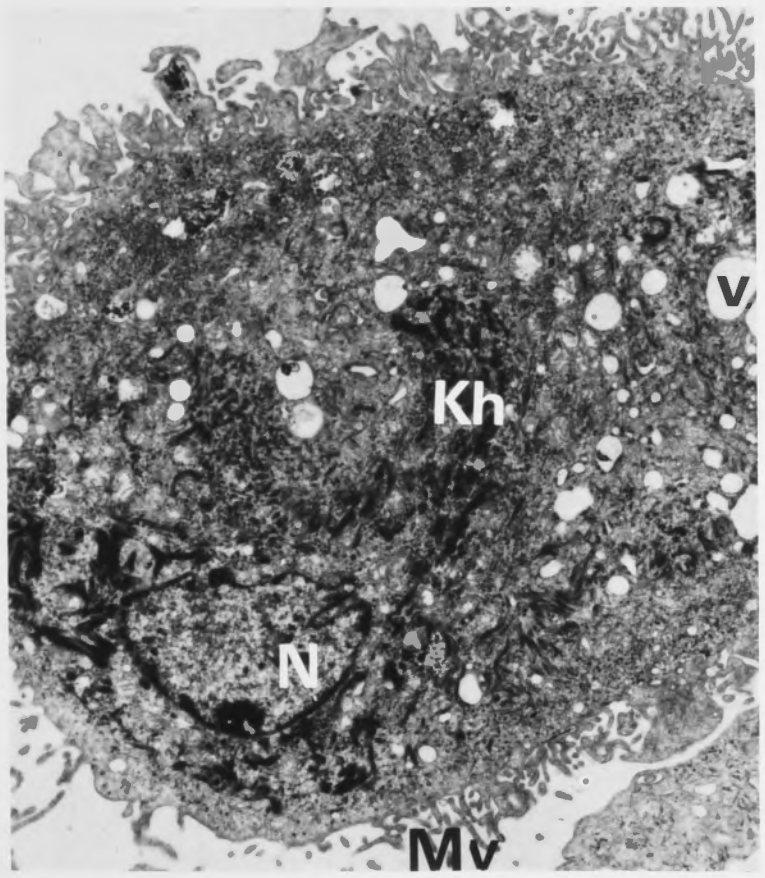
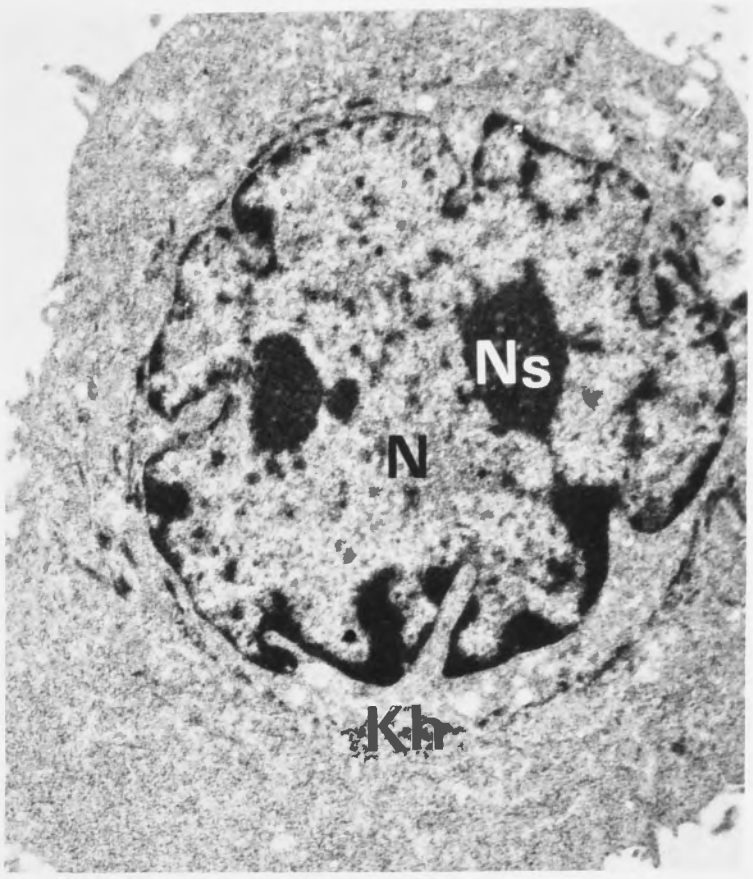

b

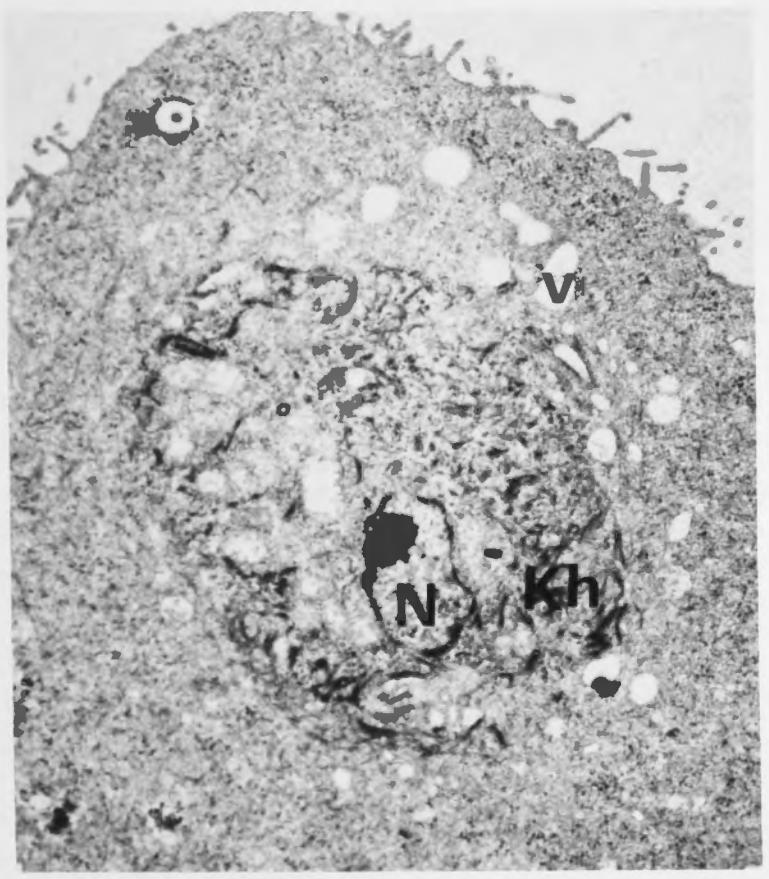
Figure 3.7 Epithelial keratinocytes obtained from cultivated tumour cell lines showing a varying degree of differentiation reflected by the amount of keratohyalin (Kh) in their cytoplasm. Note the distribution and location of keratohyalin (Kh) around the nucleus (N).

nucleolus (Ns), microvilli (Mv), vacuole (V).

(magnification X11,000)


V
Kh
N
Mv


Ns
N
Kh


V
N
Kh

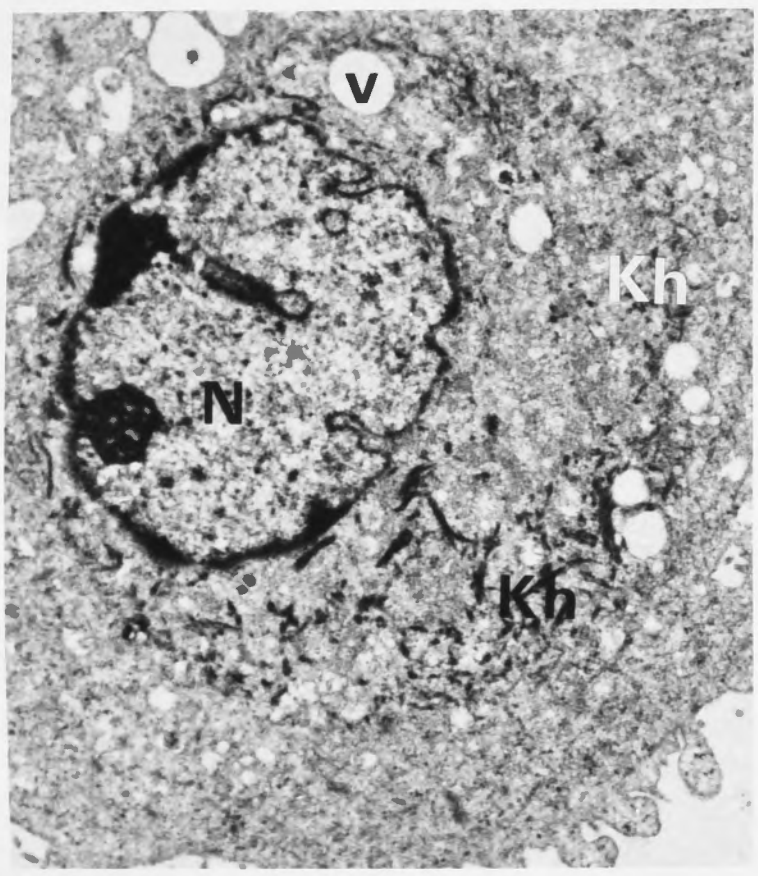

V
Kh
N
Kh

Figure 3.8 An epithelial keratinocyte showing few keratohyalin (Kh) granules distributed around the golgi (G) area and the nucleus (N). A centriole (C) is cut in section close to the nucleus (N).

nucleolus (NS).

(magnification X27,000)

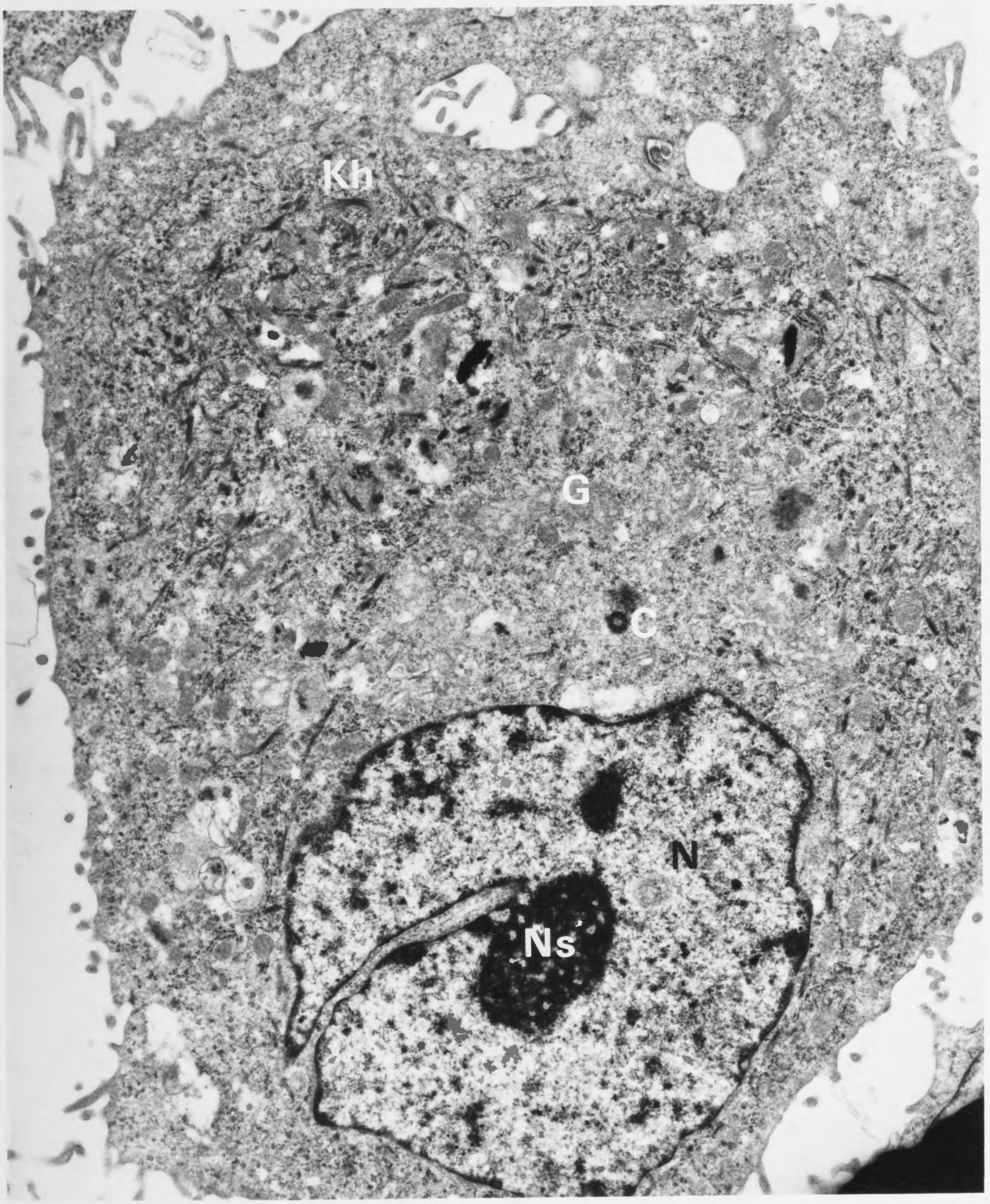

Kh
G
C
N
Ns

Figure 3.9 An epithelial keratinocyte showing a nucleus (N) with a prominent nucleolus (Ns). The keratohyalin (Kh) is distributed around the nucleus (N). Some vacuoles (V) are seen in the cytoplasm.

(magnification X35,000).

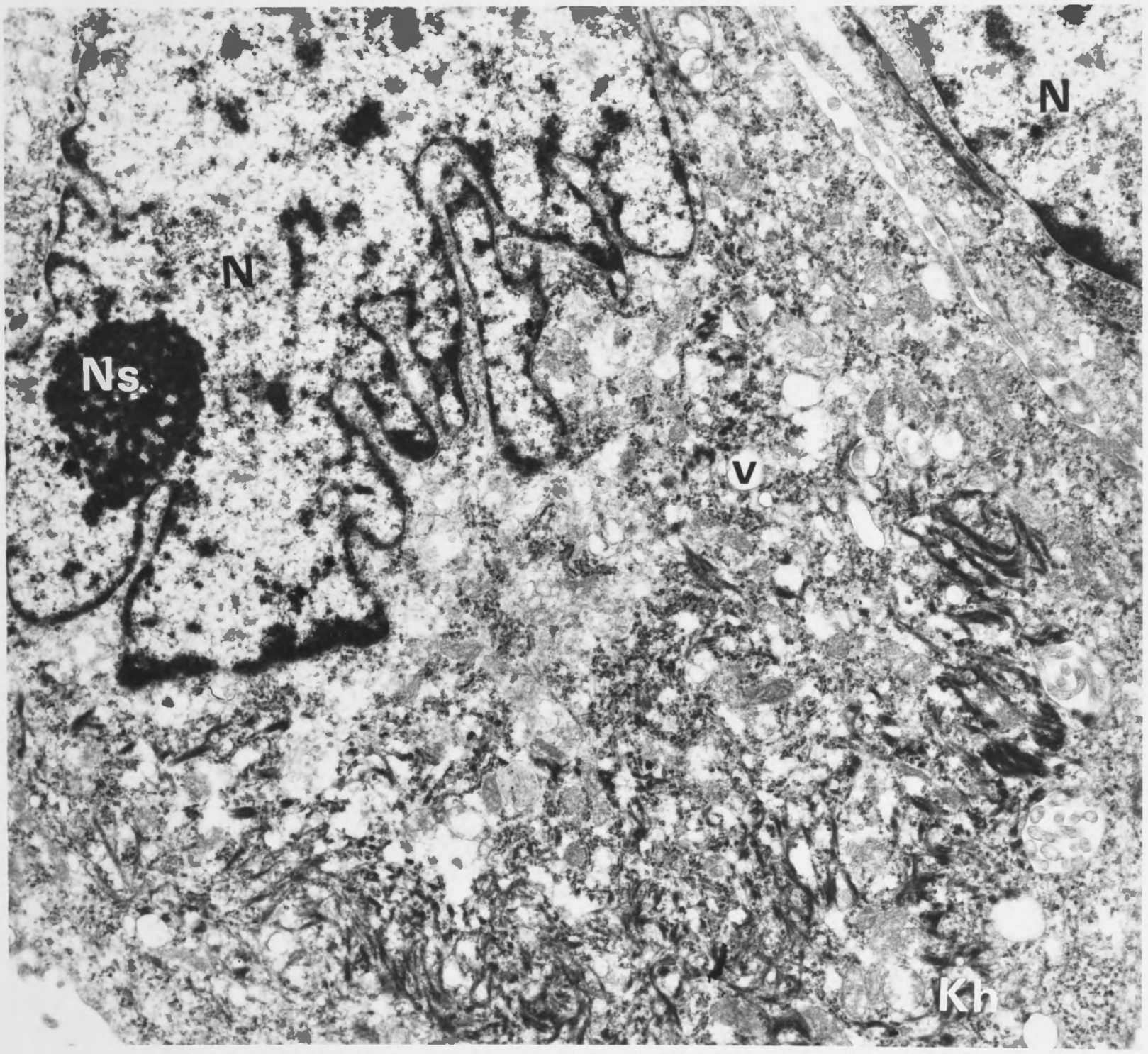

N
N
Ns
V
Kh

Figure 3.10 A more differentiated keratinocyte with the cytoplasm of the cell filled with tonofibrils (Tf) arranged in bundles. Polyribosomes (Pr), mitochondria (M) and the nucleus (N). (magnification X77,000)

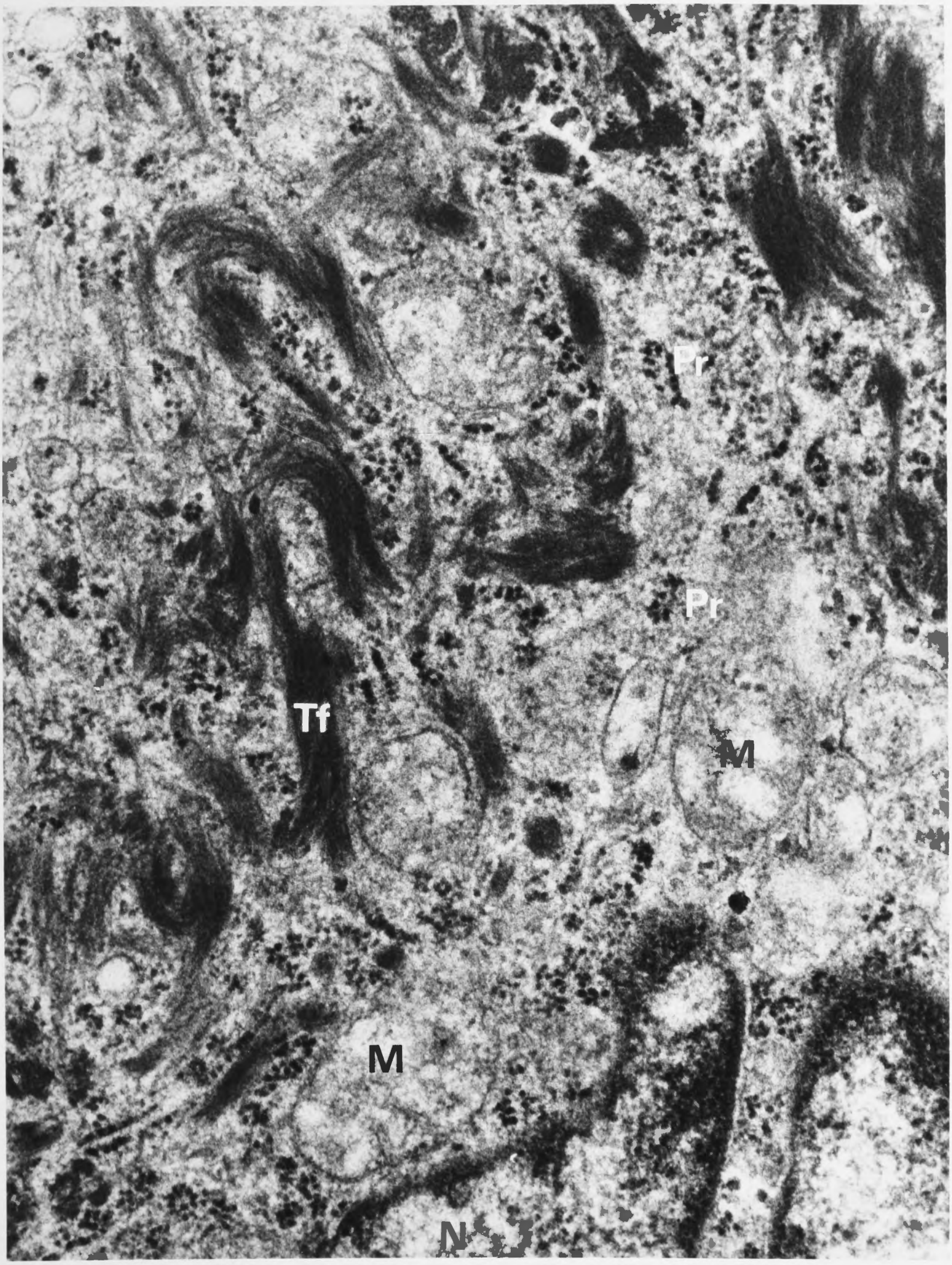
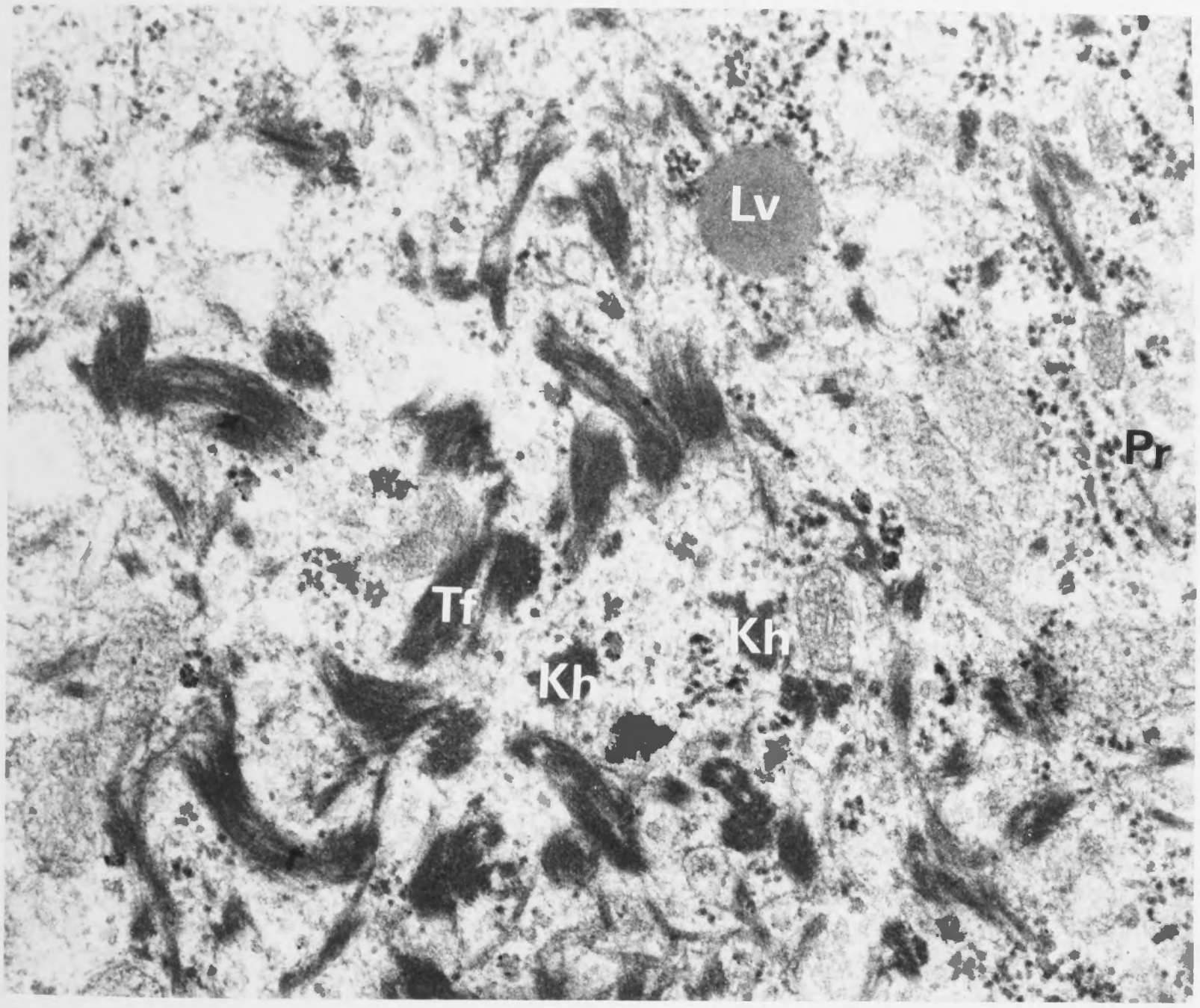

Pr
Pr
Tf
M
M
N

Figure 3.11 A differentiated epithelial keratinocyte showing the presence of large amounts of keratohyalin (Kh) and tonofibrils (Tf). Polyribosomes (Pr) are also seen scattered in the cytoplasm of the cell. Lipid vacuole (Lv).

(magnification X48,000)

Figure 3.12 A differentiated epithelial keratinocyte showing the arrangement of tonofibrils (Tf) in a circumferential ring near to the cell membrane following the dissolution of the nuclear membrane (Nm). Melanin granules (Mg) are also seen in the cytoplasm of these cells.

(magnification X35,000)


Lv
Pr
Tf
Kh
Kh

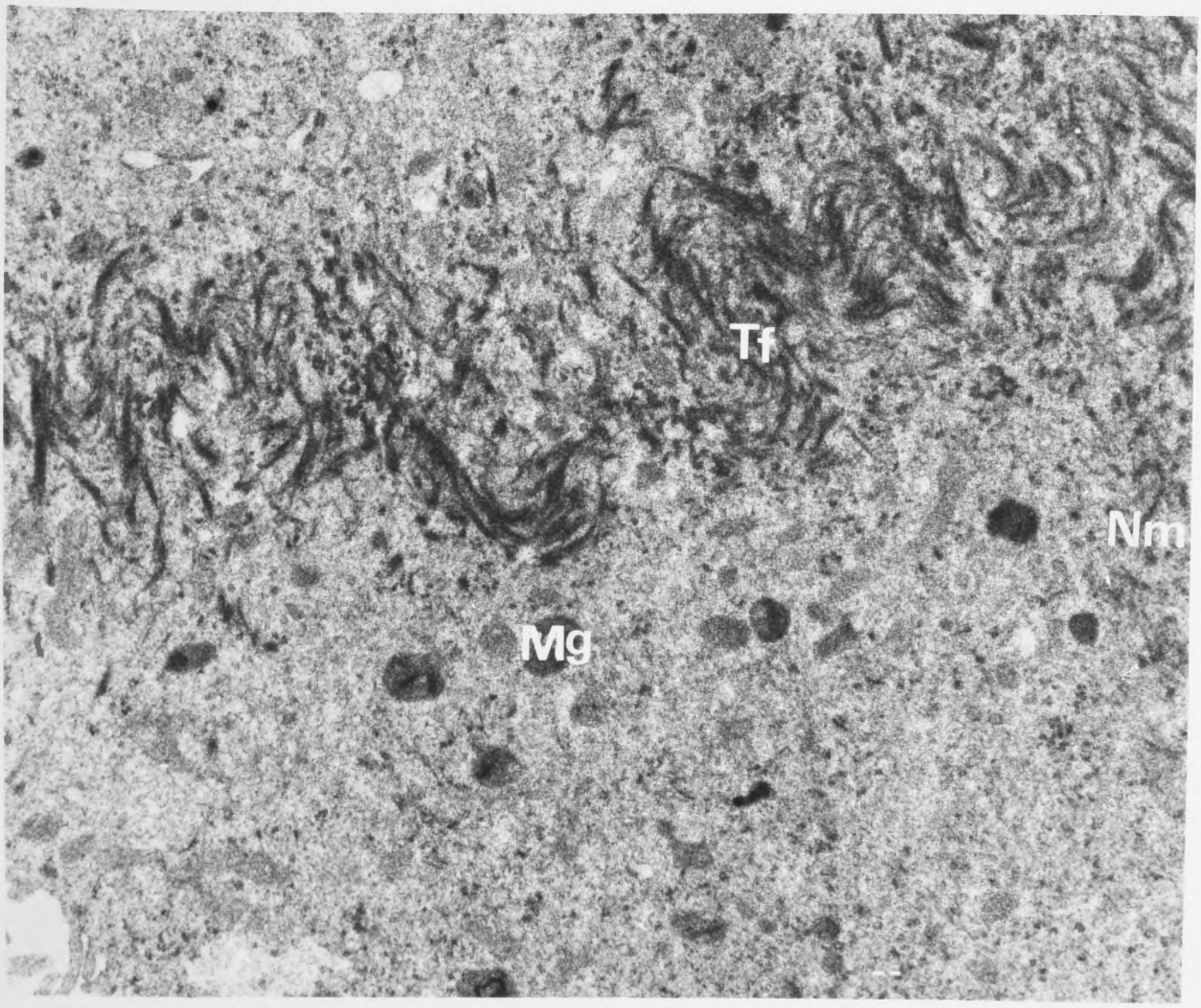
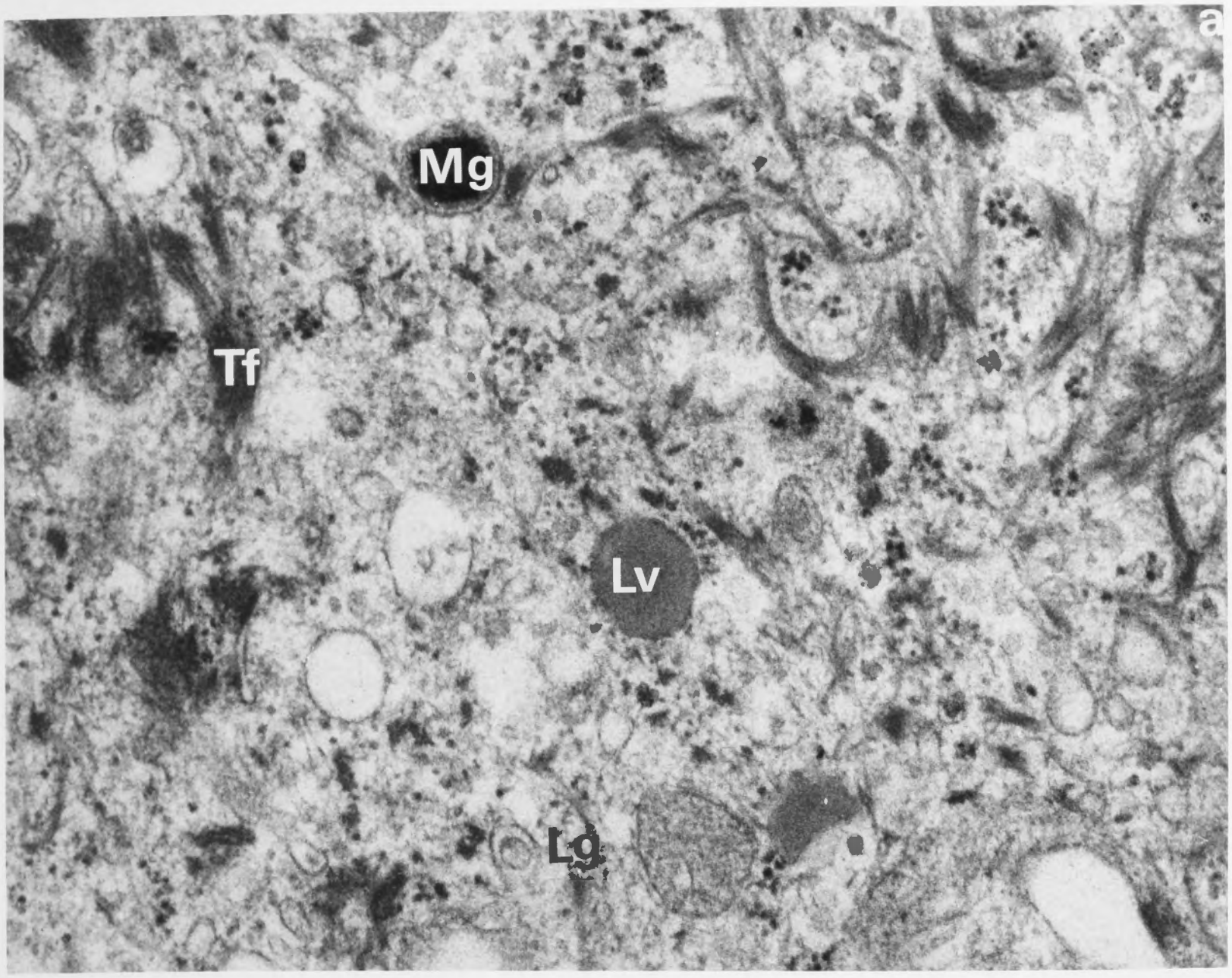

Tf
Nm
Mg

Figure 3.13 (a and b) Examples of epithelial keratinocytes showing lamellar granules (Lg), tonofibrils (Tf), keratohyalin (Kh), melanin granules (Mg) and lipid vacuoles (Lv), nucleus (N), nucleolus (Ns).

(a) magnification X48,000

(b) magnification X32,000


a
Mg
Tf
Lv
Lg

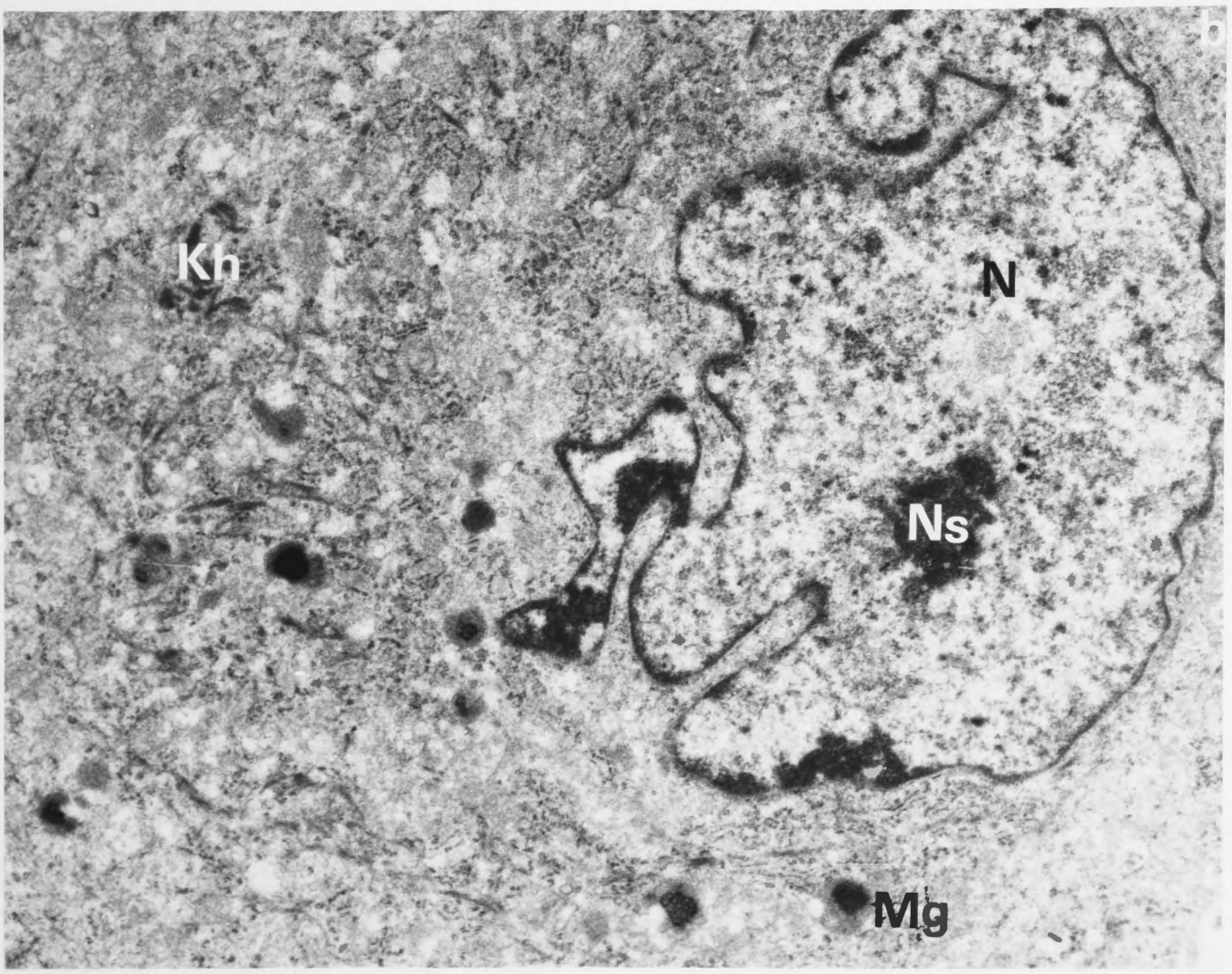

b
Kh
N
Ns
Mg

Figure 3.14 Epithelial keratinocyte showing large numbers of polyribosomes (Pr) in the cytoplasm arranged in spirals and clusters. A nuclear pore (Np) is seen in the nuclear membrane (Nm). nucleus (N), nucleolus (Ns).
(magnification X71,000)

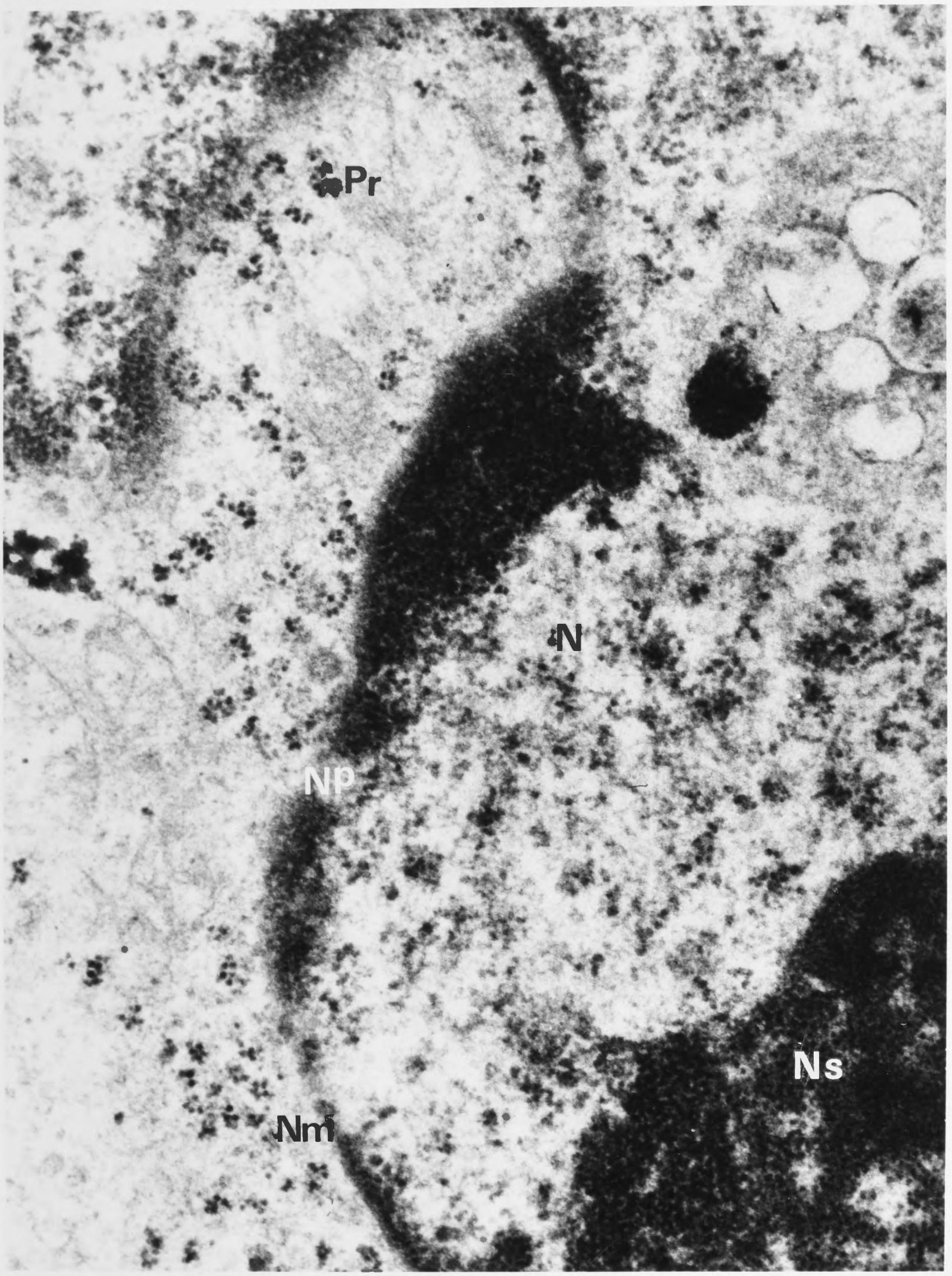

Pr
N
Np
Ns
Nm

Figure 3.15 A basal epithelial cell, showing the presence of large numbers of glycogen (Gl) granule clusters in the cytoplasm. A few tonofibrils (Tf) are seen in the cytoplasm. nuclear membrane (Nm), nucleus (N).

(magnification X24,000)

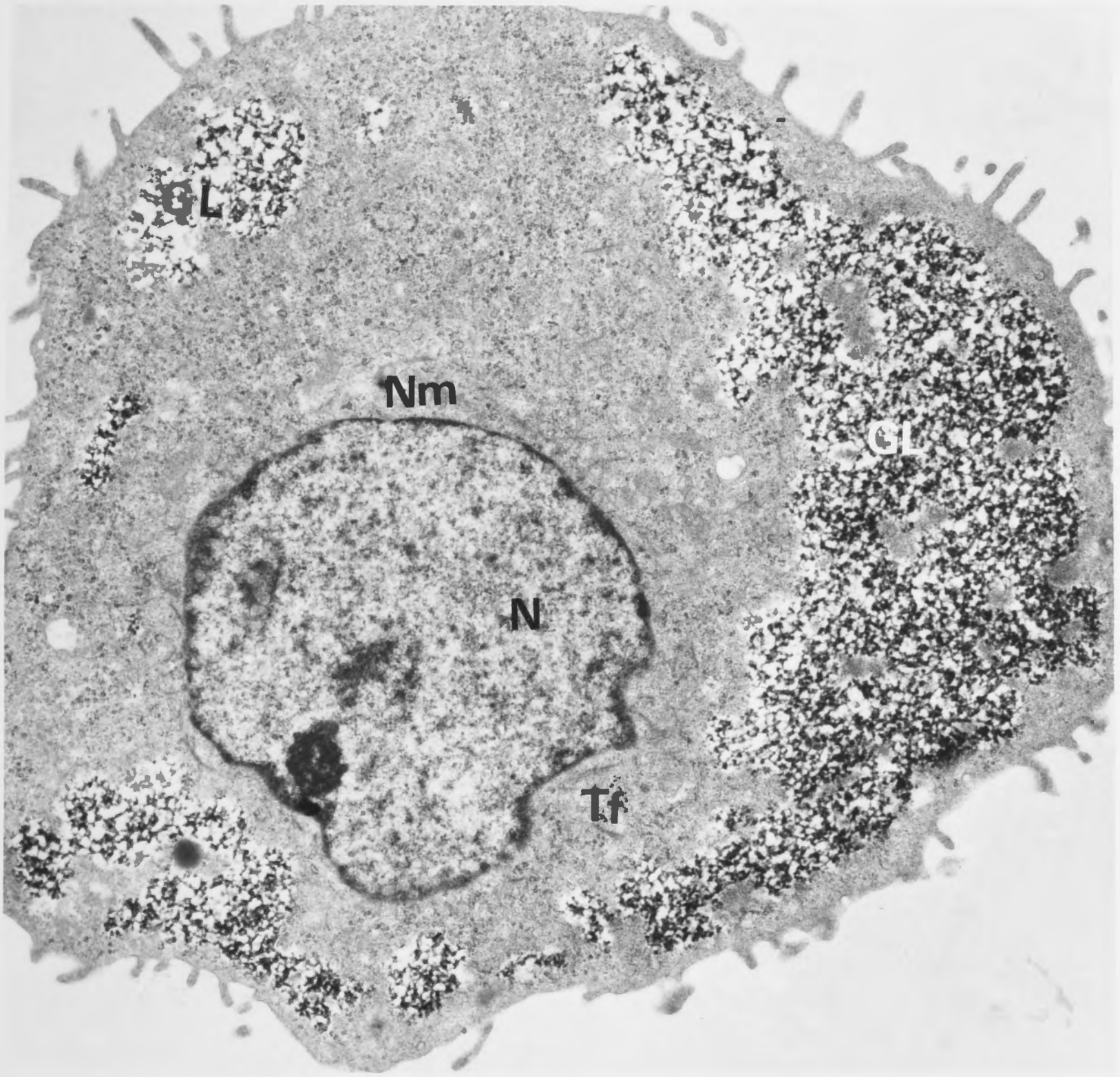
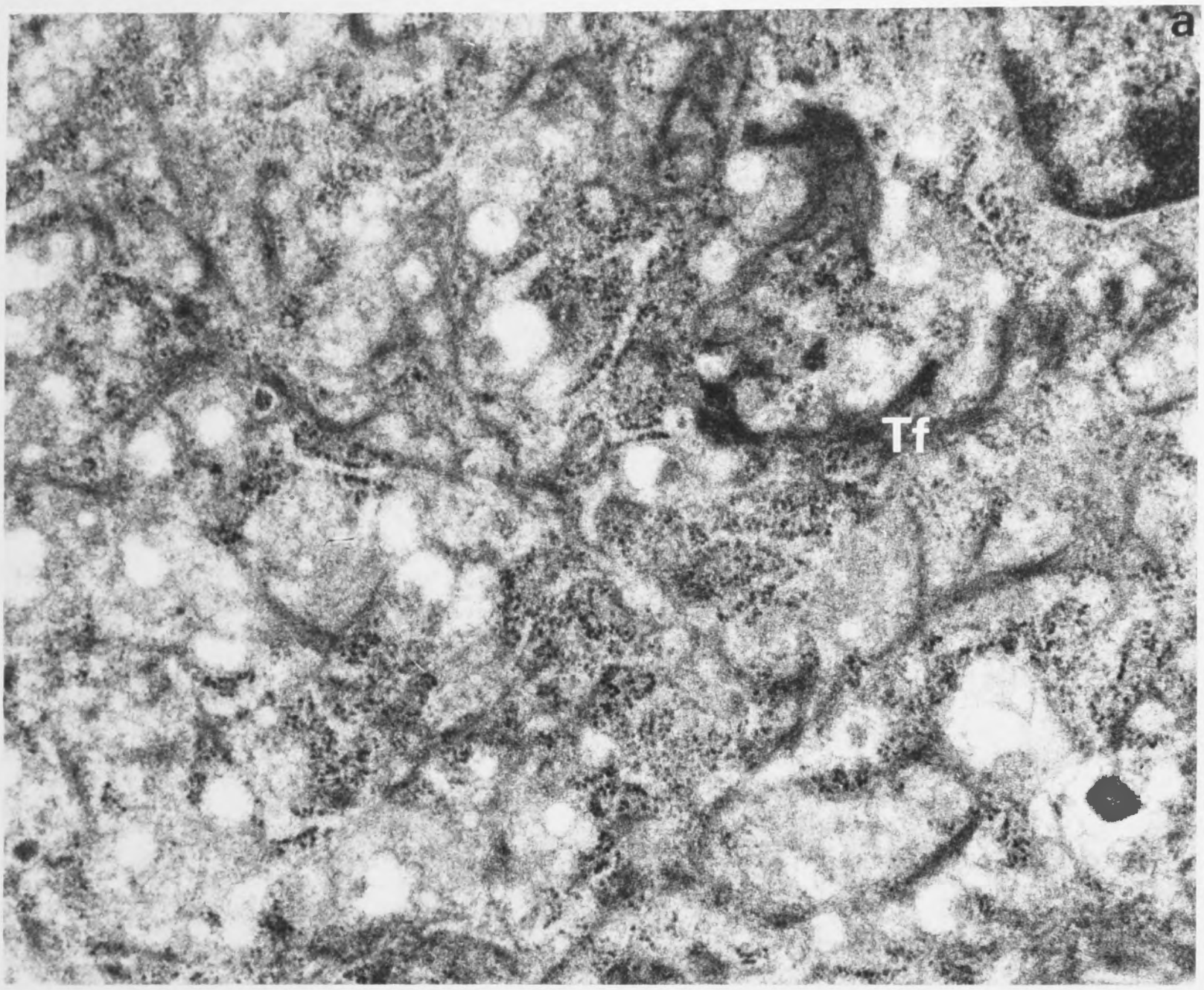

GL
Nm
GL
N
Tf

Figure 3.16a A basal epithelial cell showing a few tonofibrils scattered throughout the cytoplasm. The tonofibrils are not arranged in bundles.

(magnification X40,000)

Figure 3.16b Two basal epithelial cells joined by a desmosome (D). The desomosomal boundaries are thickened and more electron dense than the adjacent plasma membrane.

(magnification X70,000)


a
Tf

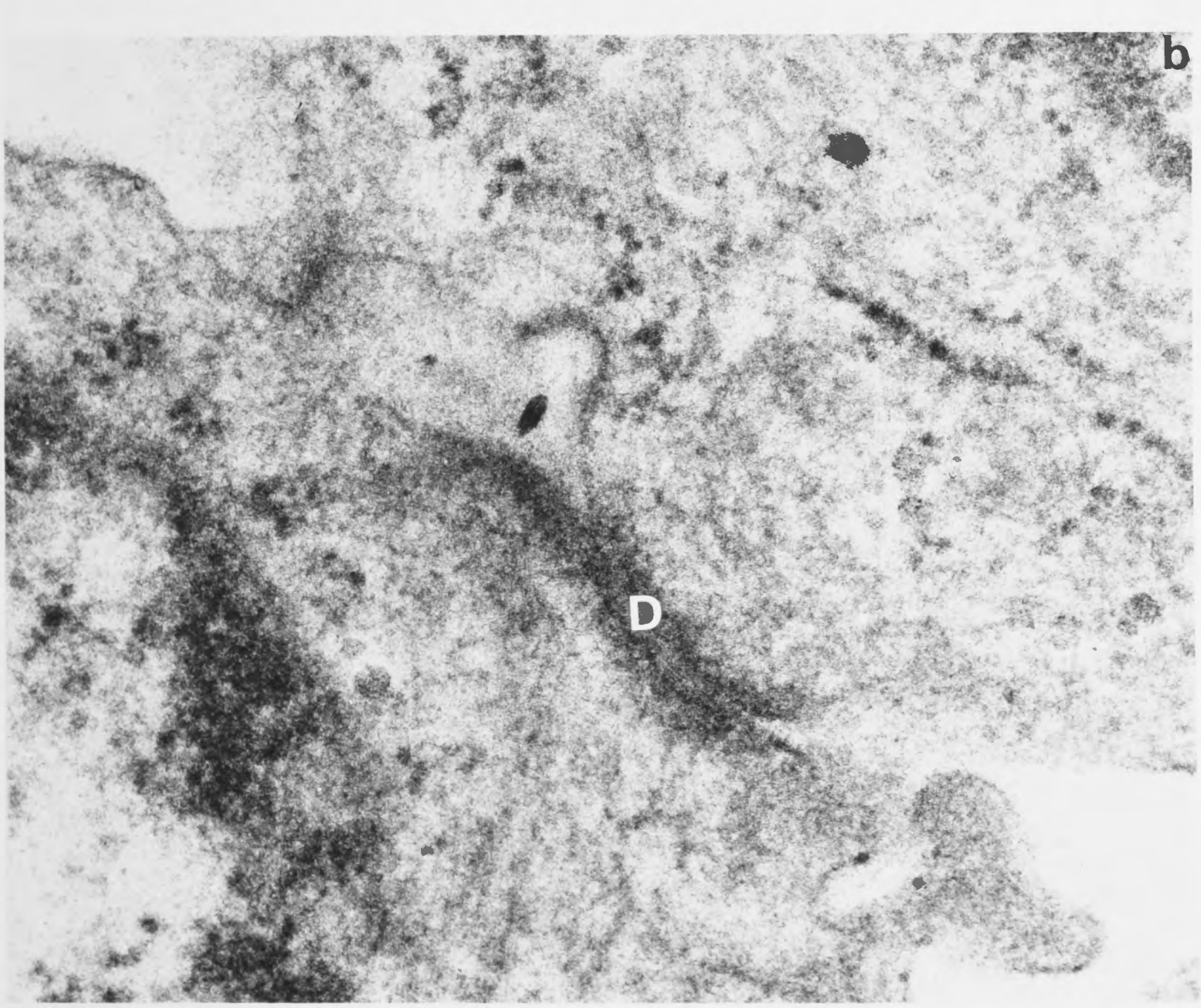

b
D

Figure 3.17 A spinous epithelial cell showing more tonofibrils and keratohyalin (Kh) in the cytoplasm than the basal cells. Some glycogen (Gl) granules and vacuoles (V) are seen in the cytoplasm of these cells.

nucleus (N), nucleolus (Ns), microvilli (Mv).

(magnification X27,000)

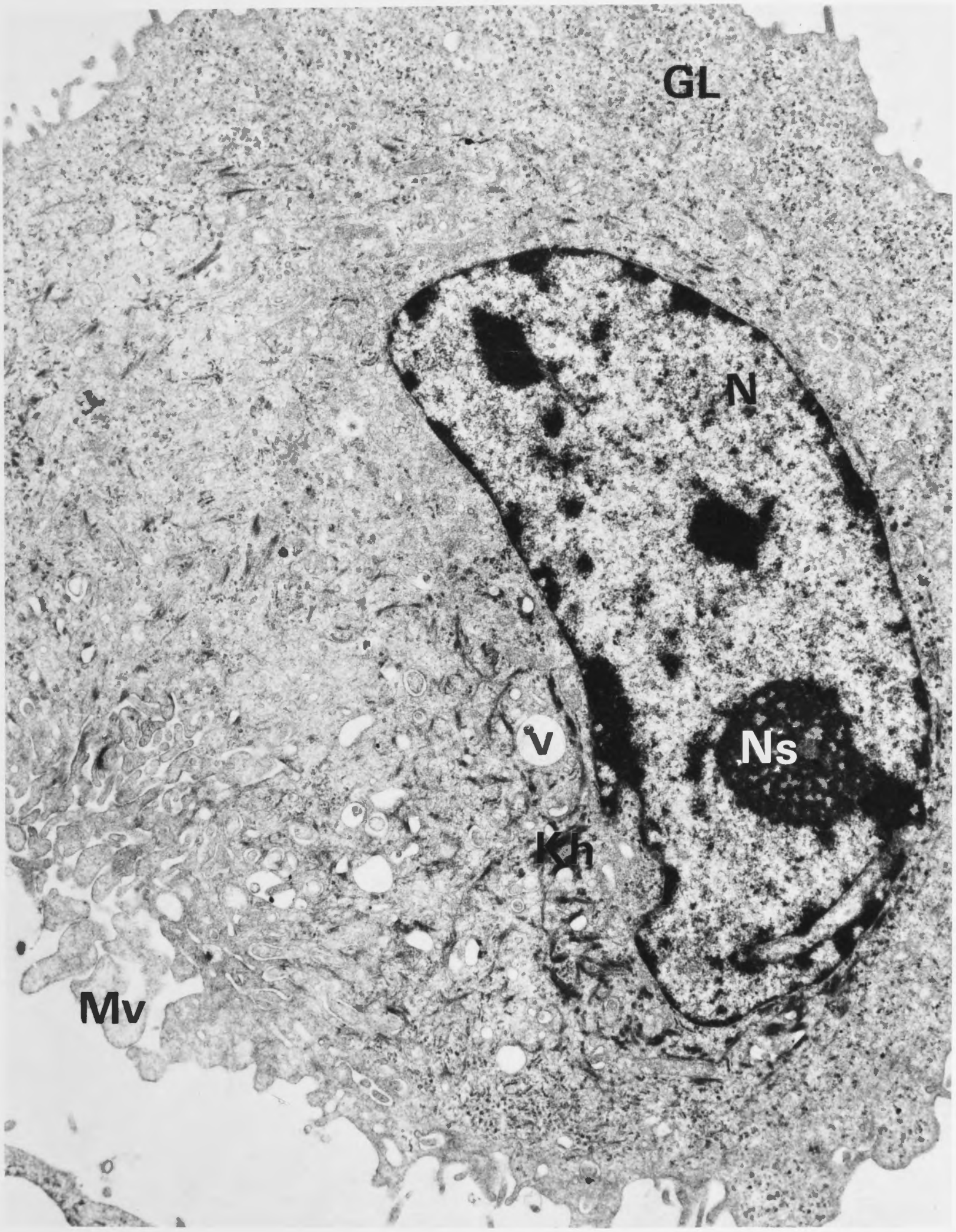
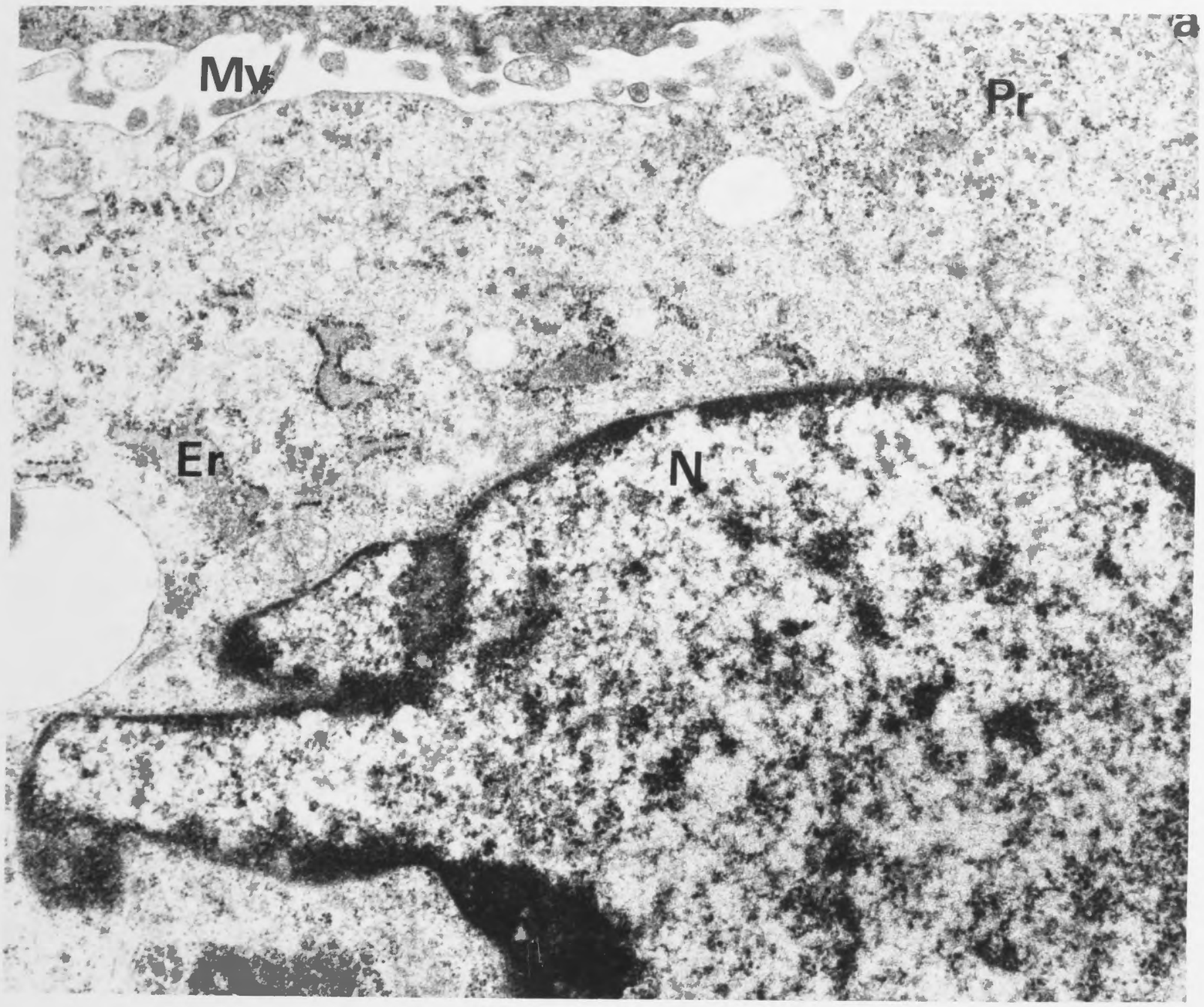
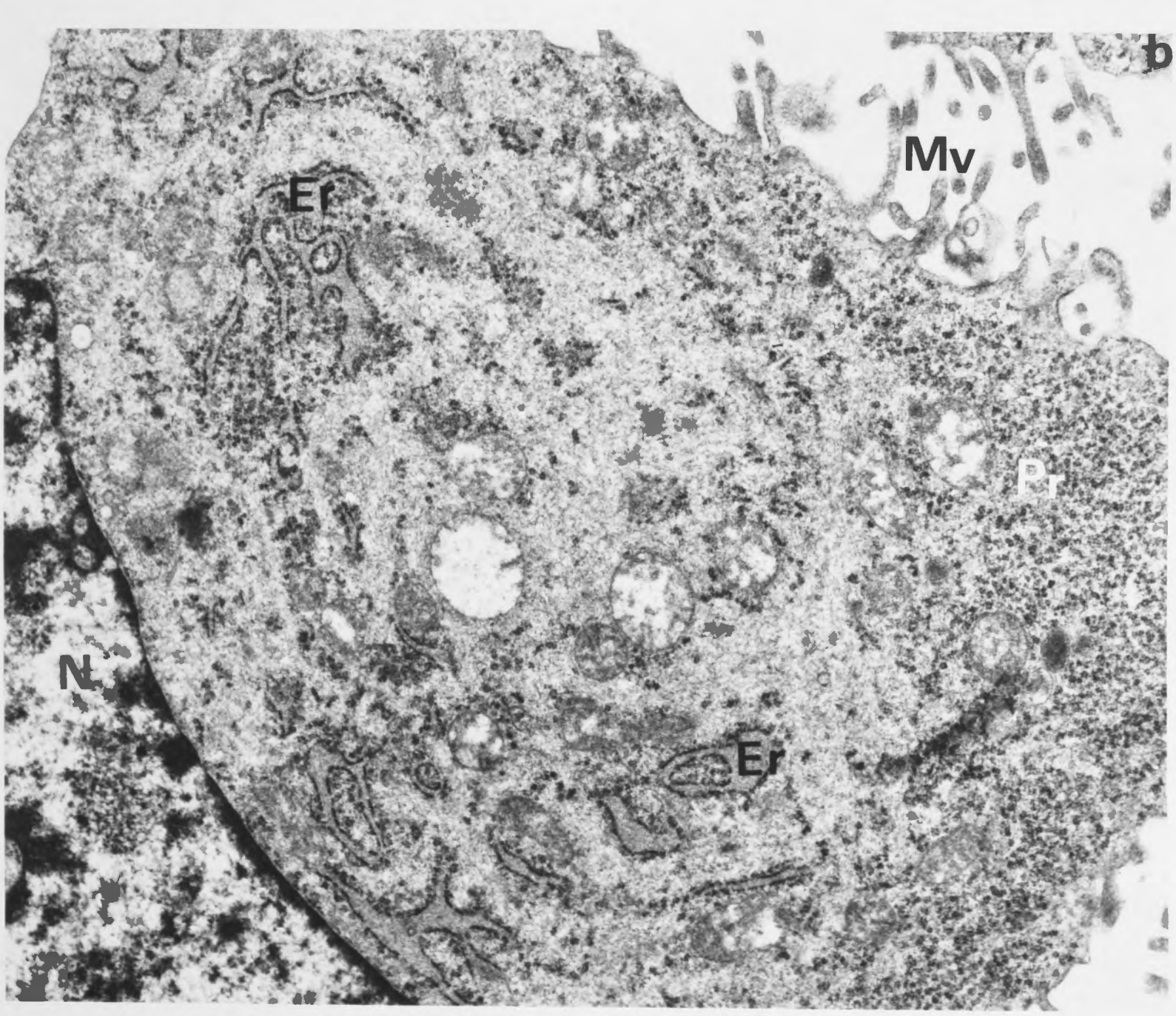

GL
N
Ns
V
Kh
Mv

Figure 3.18 (a and b) The tumour epithelial cultures contained other cells with profiles of endoplasmic reticulum (Er) and polyribosomes (Pr) in their cytoplasm. The identity of these cells was uncertain.

microvilli (Mv), nucleus (N).

(magnification X35,000)


a
My
Pr
Er
N


b
Mv
Er
Pr
N
Er

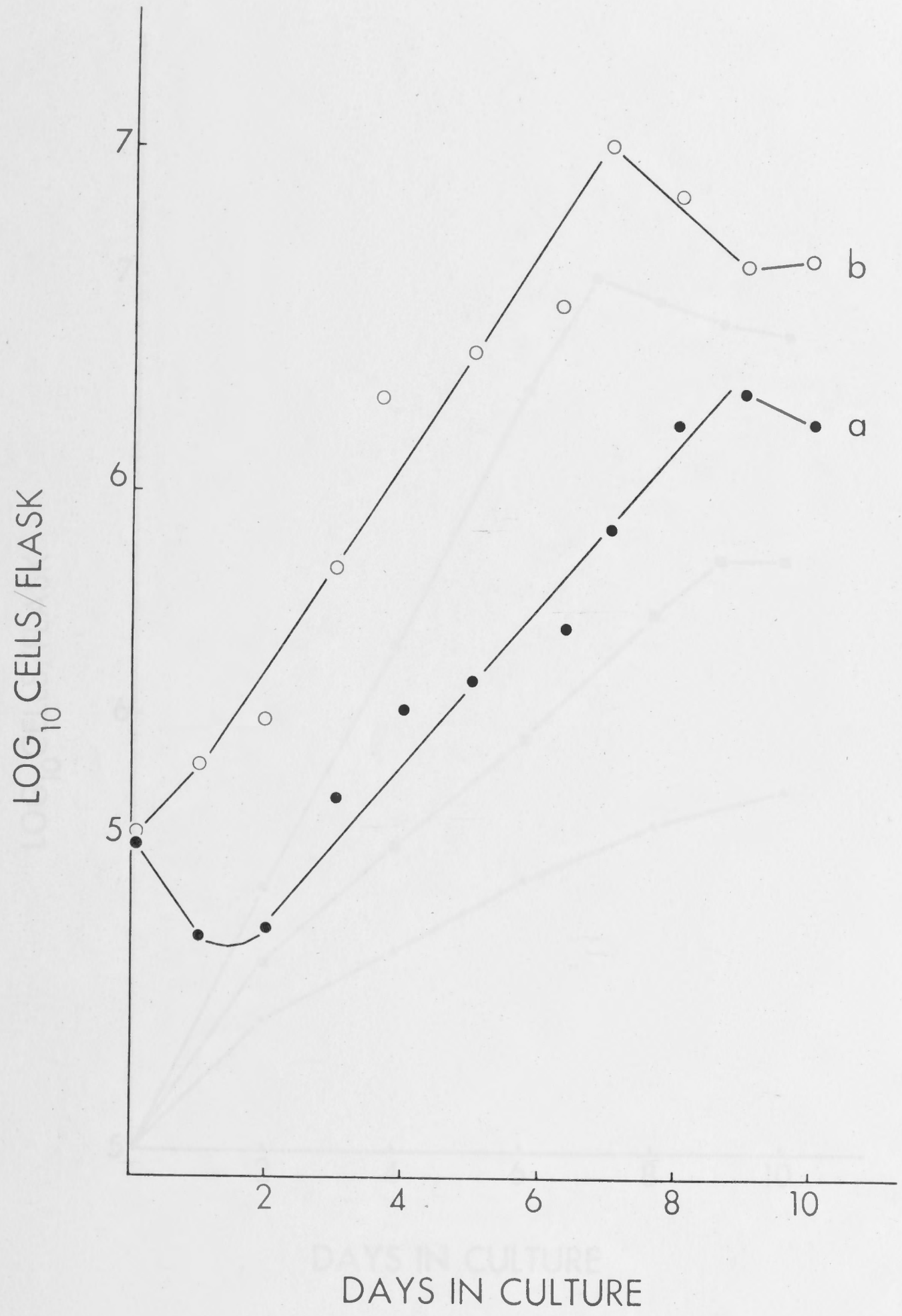


Figure 3.19 Growth rates of two different tumour cell lines at the 12th subculture level *in vitro*. F6 (O — O) showing the pattern of growth which did not have a lag phase and F18 (● — ●) represents the type of growth which had a lag phase.

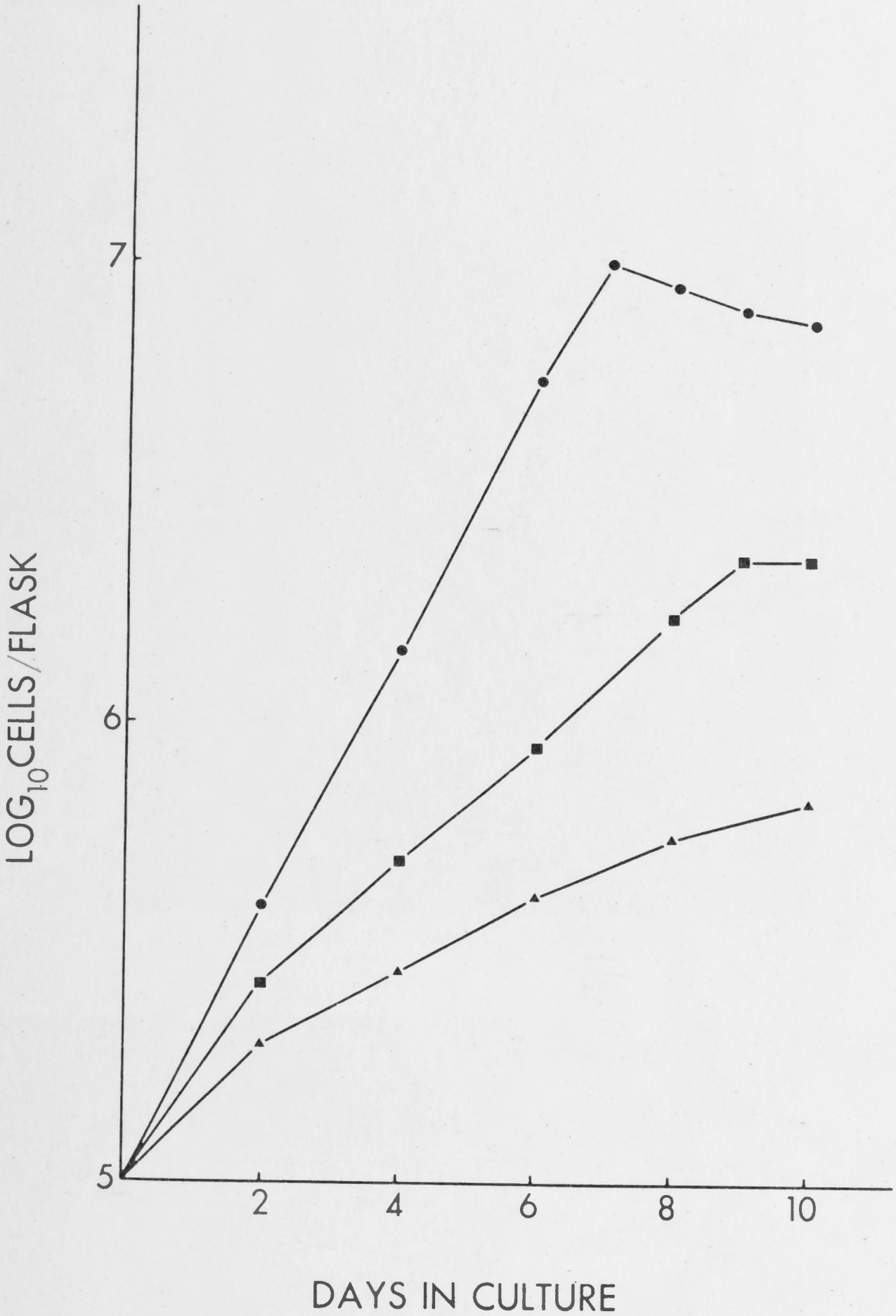


Figure 3.20 Variations in the growth rate of the tumour cell line F43 after subcultivation *in vitro*. Growth rates were determined at the 6th (▲ — ▲), 10th (■ — ■) and 20th (● — ●) subculture level.

Figure 3.21 This figure demonstrates the expansive growth pattern of the tumour F18 in the original host.

Figure 3.22 The growth pattern of tumour F18 in the nude mouse. As seen, the tumour is well circumscribed and can be detached easily from the body wall (30 days after transplantation, 2nd passage).

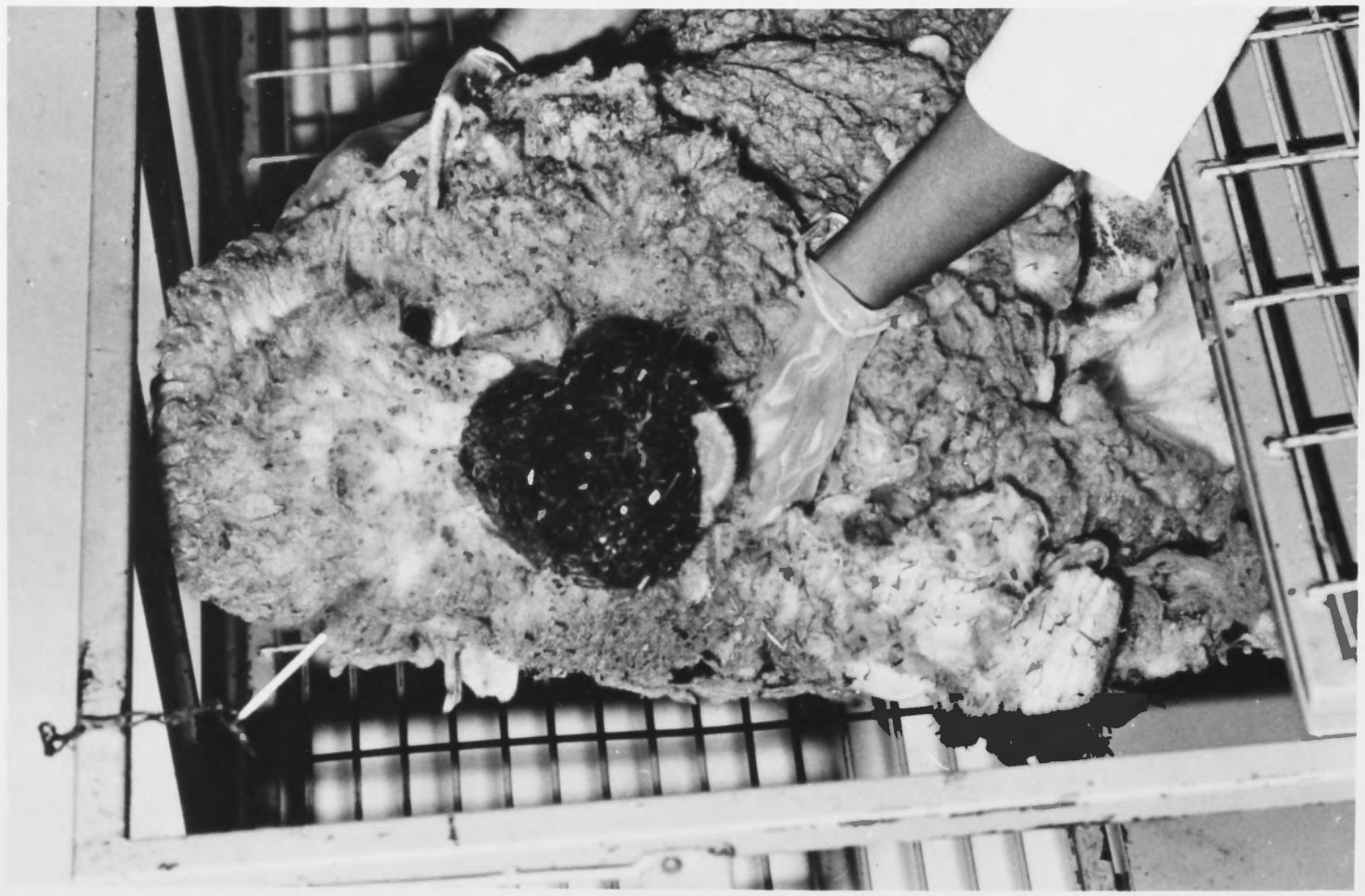
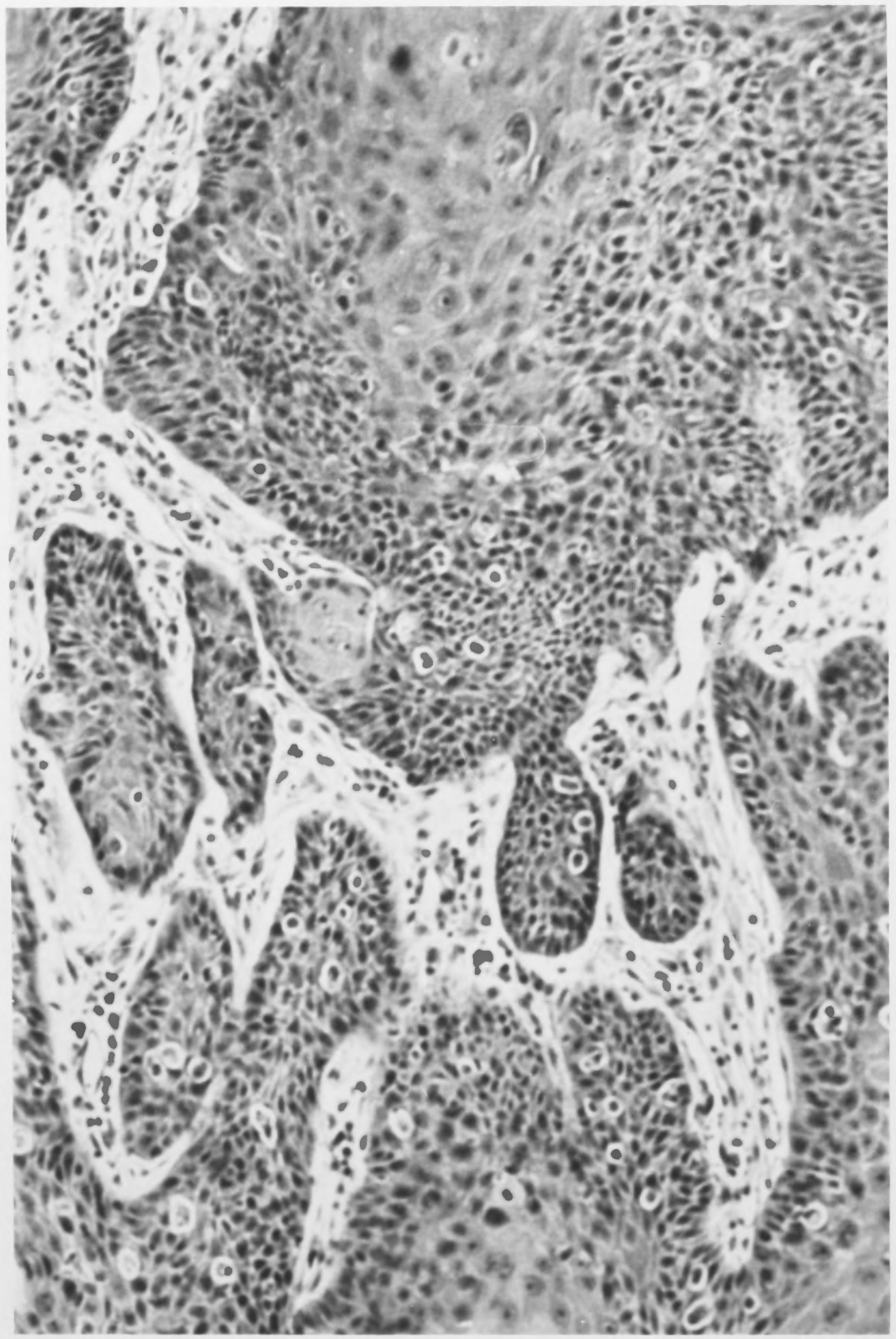
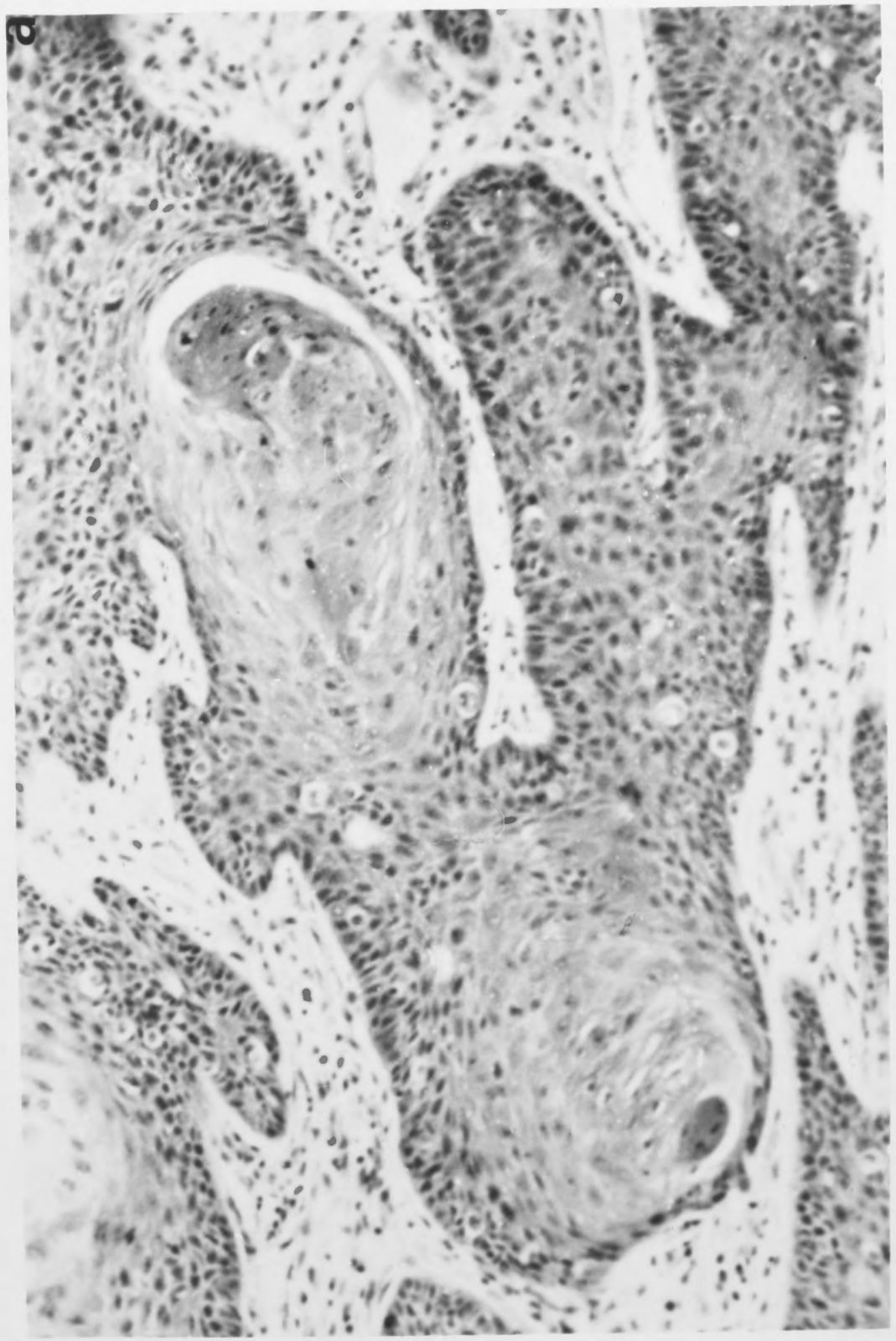


Figure 3.23 The histological appearance of tumour F18 in the primary host (sheep). This is a squamous cell carcinoma with keratinized centres (magnification X125).

Figure 3.24 The histologic appearance of tumour F18 transplanted into the nude mouse:

(a) The morphology is similar to that seen in the sheep (magnification X125).



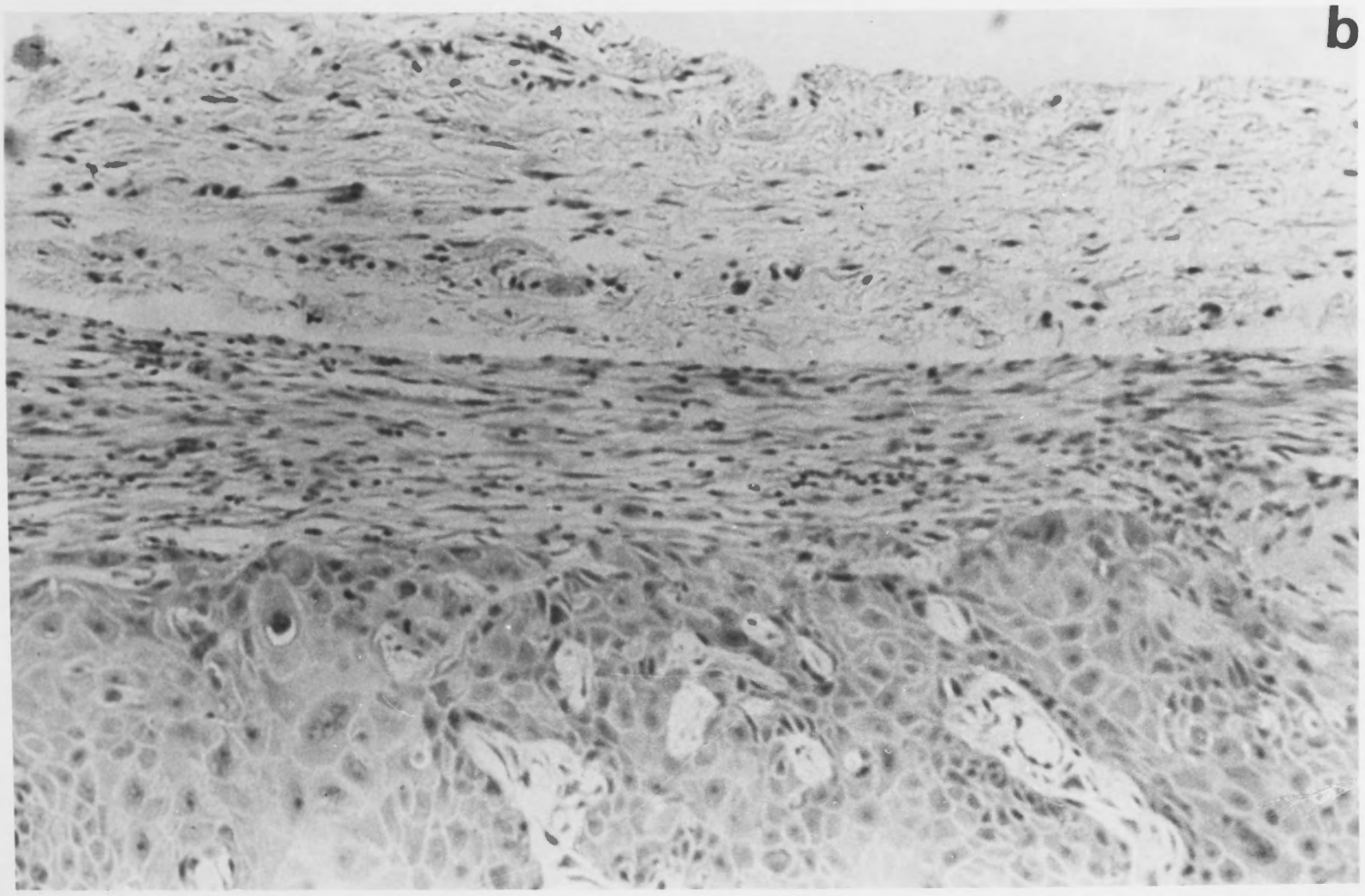


(b) Thick layers of stromal elements surrounding the tumour.

(magnification X125).

(c) The presence of necrotic centres within the tumour

(magnification X50).


b

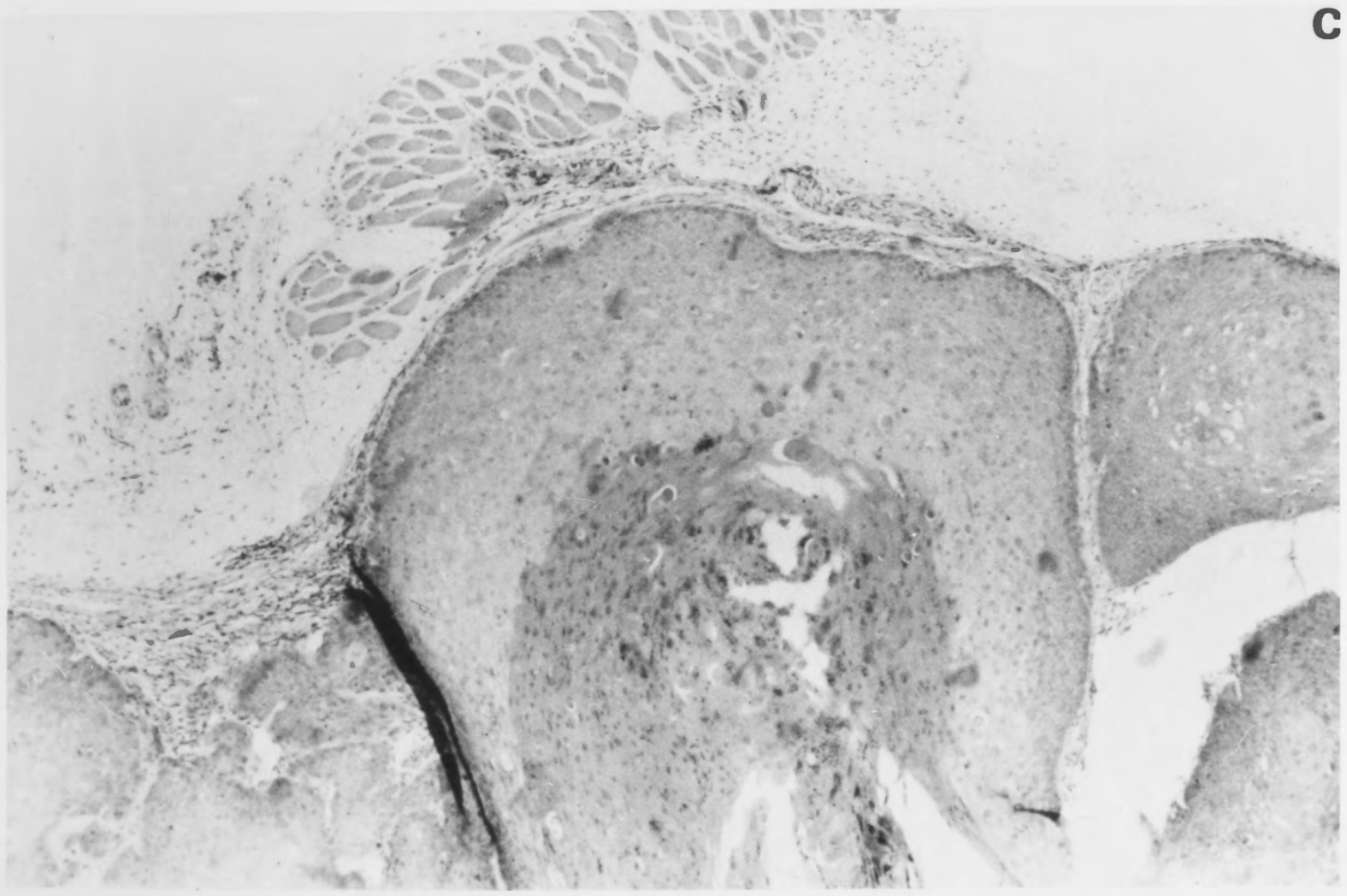

c

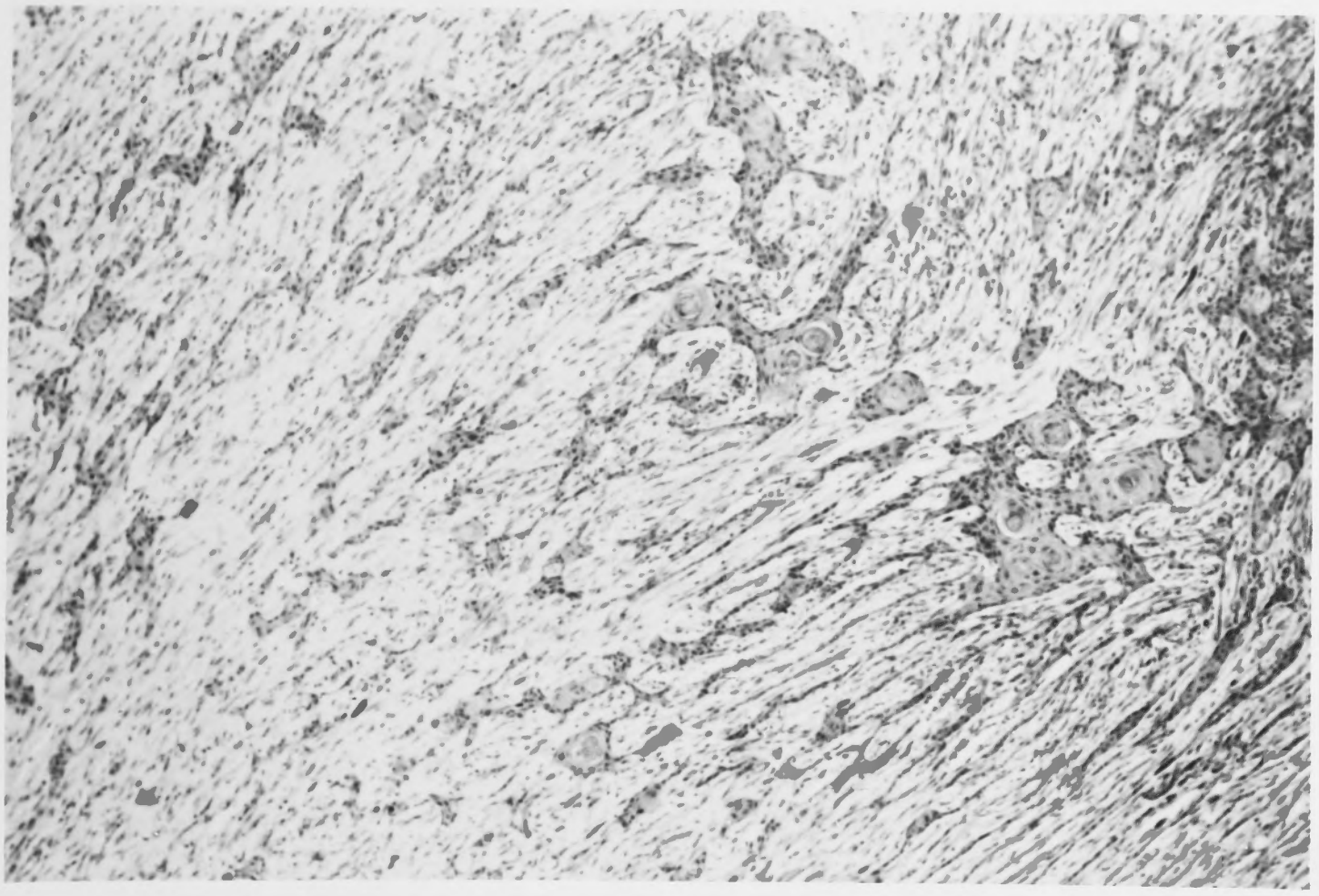
Figure 3.25 The morphological appearance of the tumour F6 in the primary host. This was a highly invasive tumour.

Figure 3.26 The growth pattern of tumour F6 in the nude mouse showing a highly vascular tumour growing attached to the body wall (60 days after transplantation, 2nd passage).

Figure 3.27 Tumour F6 showing finger-like projections of tumour tissue. These processes were found on the part of the tumour adjacent to the body wall, and represent extensions of tumour cords which subsequently invaded the muscular layer (mouse sacrificed 90 days after transplantation).

Figure 3.28 The histological appearance of tumour F6 in the primary host (sheep), note the spindle-like appearance of the cells, the presence of a few keratin pearls scattered throughout the tumour and the large amounts of fibroblastic tissue (magnification X50).

Figure 3.29 The histological appearance of the tumour F6 transplanted into the nude mouse as compared to that in sheep showing a similar cell morphology, a few keratin pearls and large amounts of stromal elements (Magnification X50).



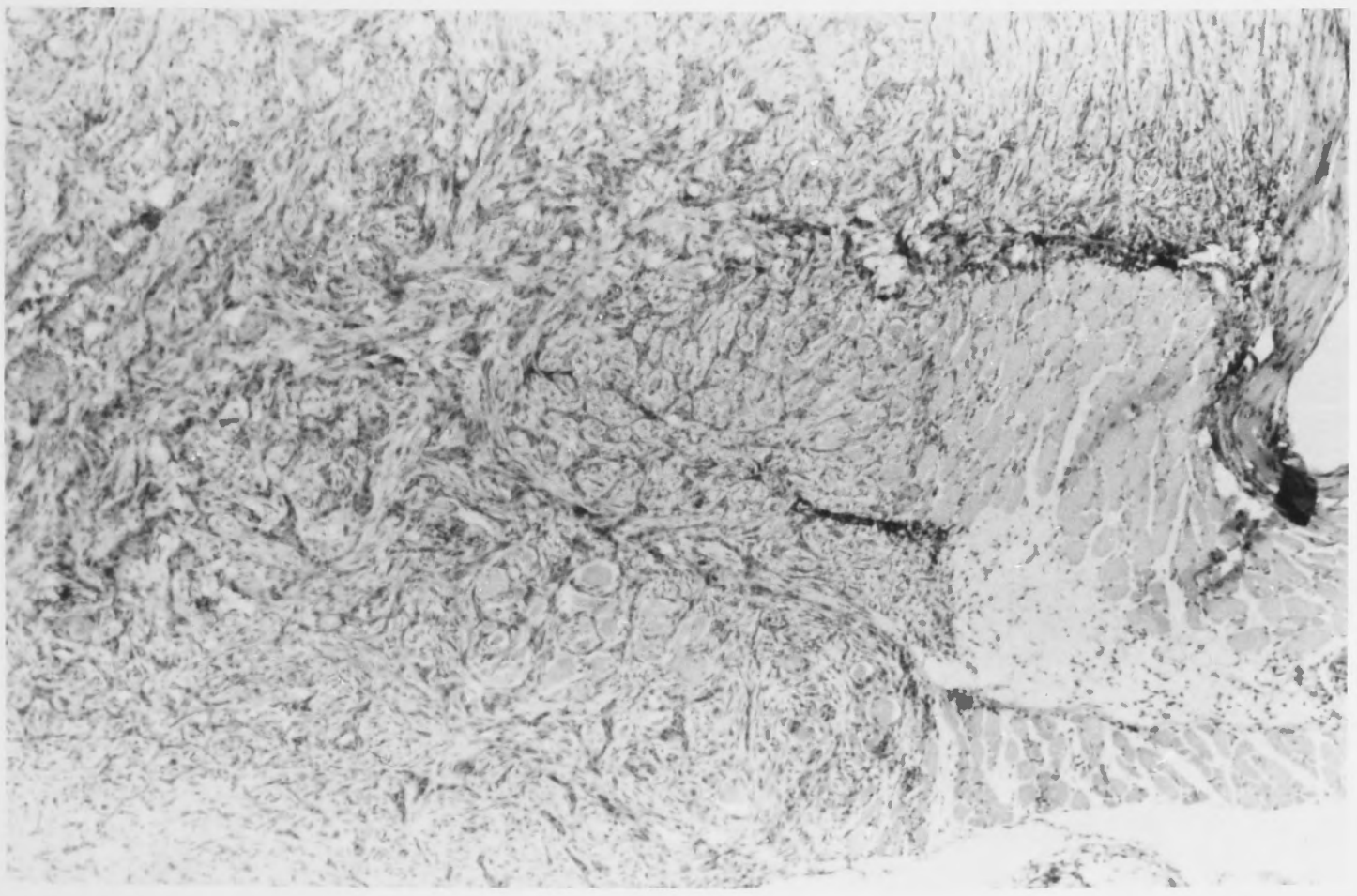
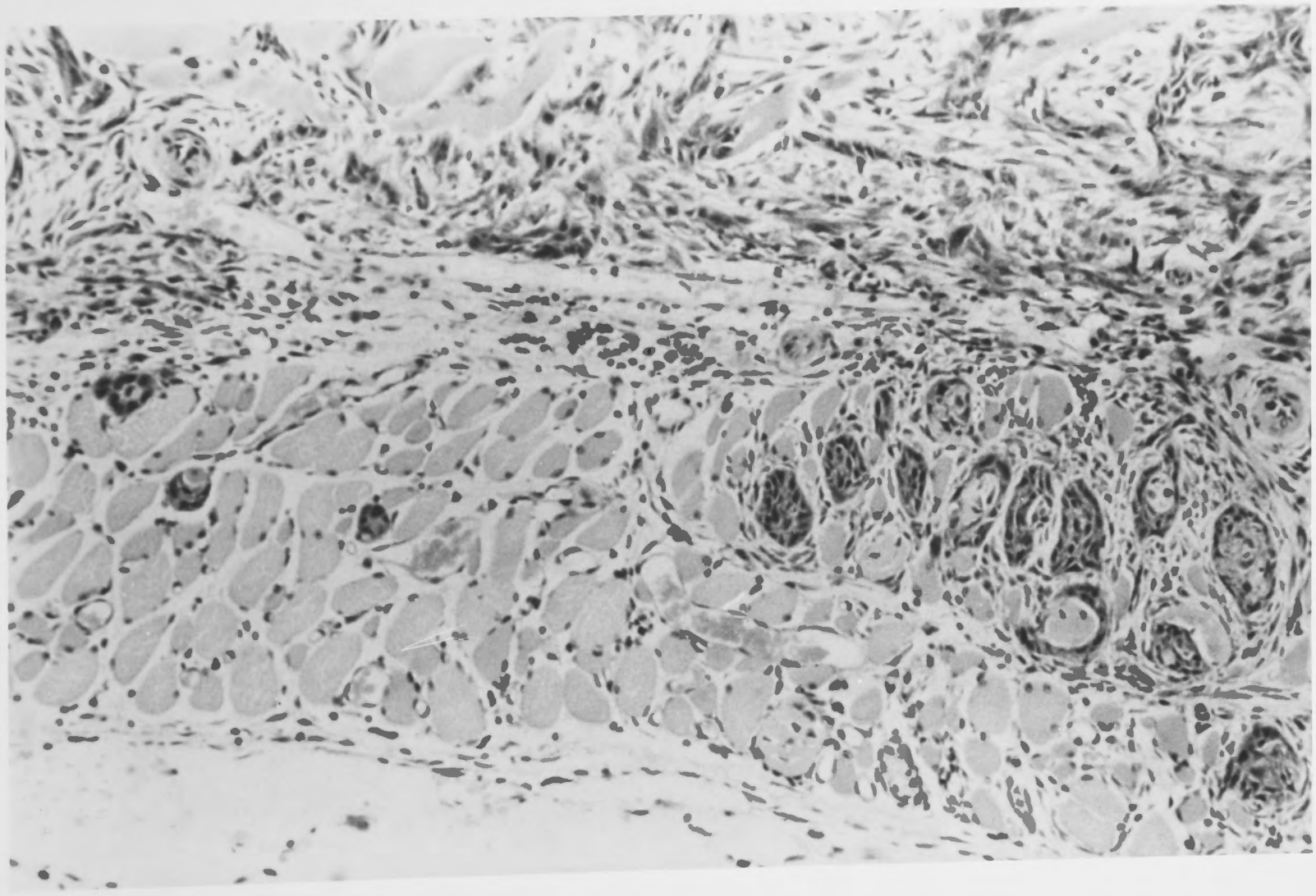
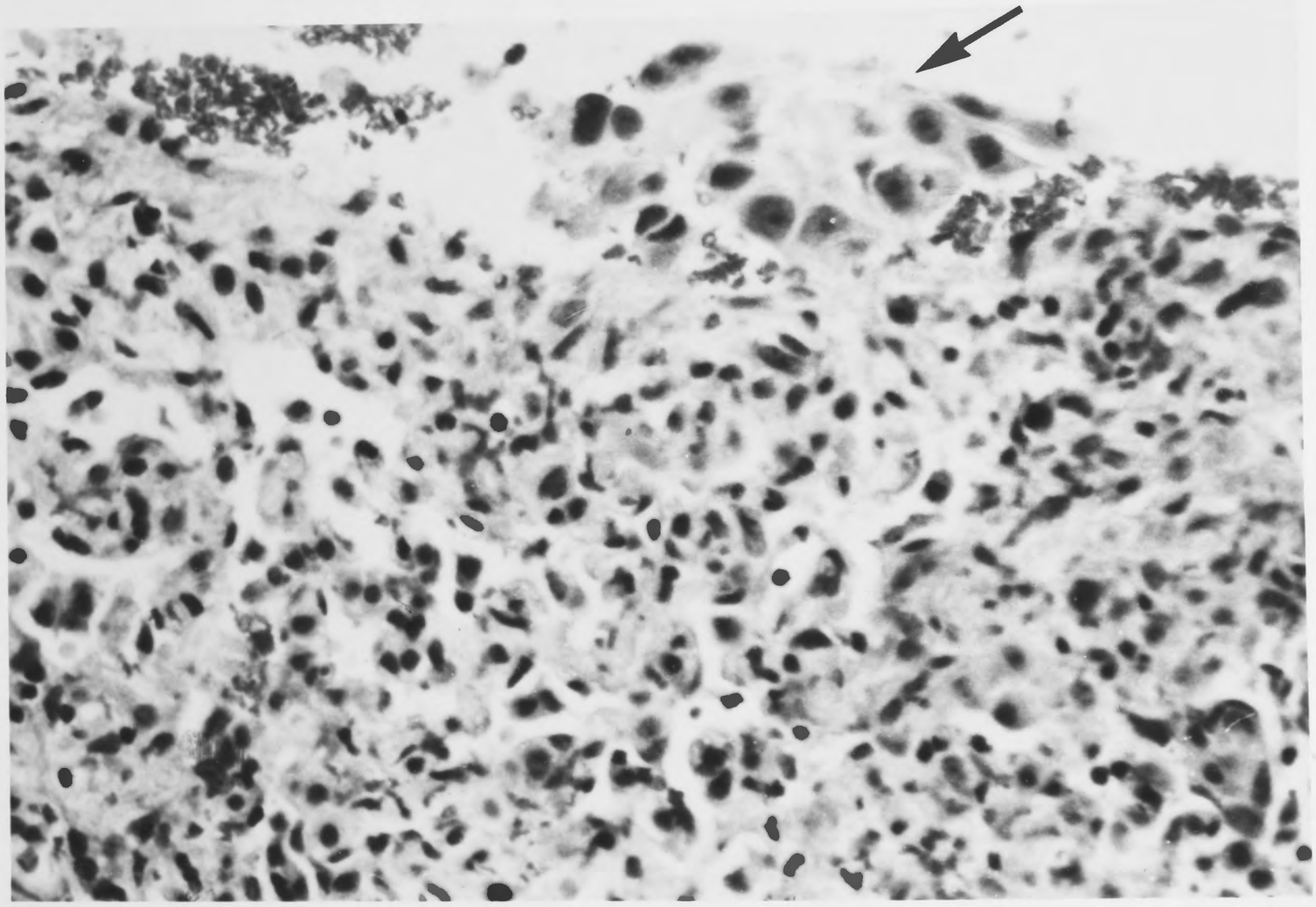


Figure 3.30 A section through tumour F6 showing the invasion of the muscle layer at the wall of the thorax by tumour cells. The individual muscle is completely surrounded by tumour cells (magnification X125).

Figure 3.31 Metastasis of tumour F6 into the tissue of the lung of the nude mouse (arrow showing tumour cells, magnification X312.5).





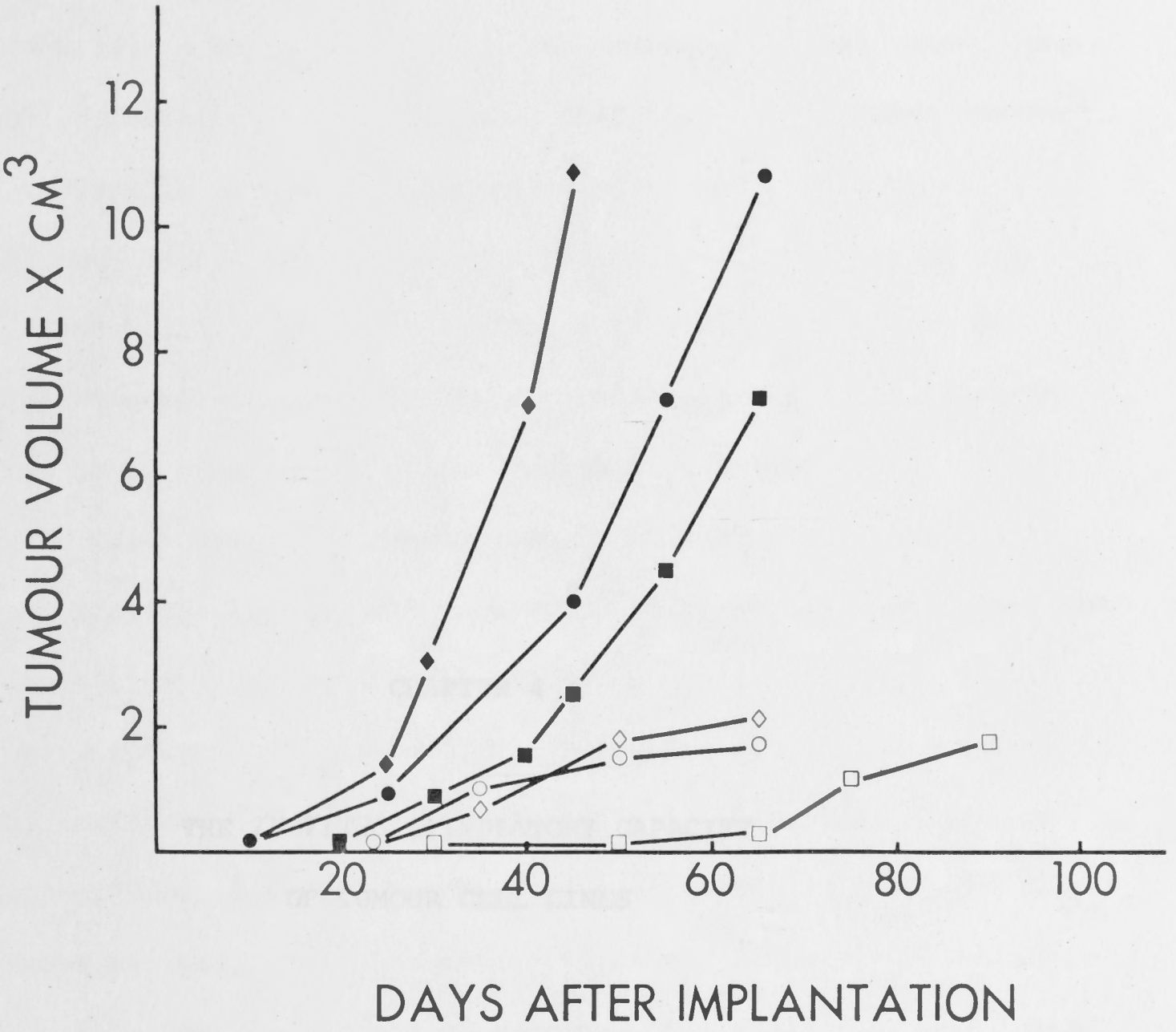


Figure 3.32 Growth rates in the nude mouse of tumours F6 at passage level 1 (□—□), 7 (○—○) and 9 (◇—◇) and F18 at the same passage levels (closed symbols).

CHAPTER 4

THE *IN VITRO* STIMULATORY CAPACITY

OF TUMOUR CELL LINES

4.1 INTRODUCTION

The role of the host's immune system in tumour control is highly complex. Many effector cells and mechanisms have been demonstrated to be involved in the response against tumours and more critically, it is now known that the host's immune system can occasionally play a role in assisting a tumour to survive. Thus although it can be assumed that the host's immune system (if it is involved in a response against a tumour) plays mainly a defensive role, under certain circumstances its activities can lead to an enhancement of the growth of the tumour.

The host-tumour interaction is further complicated by the fact that the tumour cell population is by no means uniform. The heterogeneity of tumour cell populations with regard to growth rates, antigenicity, immunogenicity, metabolic characteristics and certain other biochemical features (Fidler 1978; Fidler and Kripke 1980) is a well established observation in a variety of tumour systems. This heterogeneity is not surprising considering that the continued growth of a tumour mass will involve a degree of differentiation and metabolic change in the tumour cells. Variant cells may arise and these will be subjected to survival pressures; only those cells suited for the environment in which they find themselves will survive. However with the continuing growth of the tumour, subpopulations of surviving cells will be more likely to possess an enhanced malignancy towards the host. It is also possible that as tumour cells evolve they come to possess an increased antigenicity relative to the host and become more likely to stimulate the host's immune system. Protection may also be afforded for the different clones of

malignant cells as a result of suppressive effects on the host's immune apparatus induced by the main bulk of the tumour.

Because of the complex nature of both the host's response and of the tumour cell population, it is virtually impossible to understand the host-tumour interaction by studying one system without regard to the other. Therefore, before analyzing the immune response of tumour-bearing sheep to their autochthonous tumours, studies were first carried out in an attempt to understand some of the native properties of the tumour cells themselves.

It has been shown by a number of investigators that cells will vary in their ability to cause proliferation in lymphocytes *in vitro* when they are mixed together. Strong stimulation is observed when allogeneic lymphocytes from genetically different individuals are cultivated together (Bain *et al.*, 1964; Bach *et al.*, 1973). Some, but not all kinds of non-lymphoid tissue cells have also been reported to stimulate lymphocyte proliferation in humans, and these include skin cells (Hirschberg *et al.*, 1973), vascular endothelial cells (Hirschberg *et al.*, 1974) and spermatozoa (Levis *et al.*, 1974, 1976). Studies on tumour cell-lymphocyte interactions have revealed a somewhat similar picture. Hardy and Ling (1969) reported that lymphoma cells are able to produce a significant stimulation of allogeneic lymphocytes while HeLa cells do not. Anderson *et al.* (1972) studied the interaction between tumour cells and allogeneic lymphoid cells in 14 different types of tumours including melanoma, breast carcinoma, osteogenic sarcoma, adenocarcinoma and the HeLa cell line. They observed significant stimulation in the lymphoid cell populations when they were mixed with all these tumour cell lines; however this stimulation was low when compared to the stimulation obtained by

cell mitogens or by allogeneic lymphocytes. Thus both normal and tumour cells seem to vary in their ability to stimulate allogeneic lymphoid cells.

Experiments described in this chapter were aimed at investigating the ability of cultivated epithelial tumour cells to stimulate a proliferative response in allogeneic lymphocytes.

4.2 THE ALLOGENEIC TUMOUR CELL-LYMPHOCYTE INTERACTION

The allogeneic tumour cell-lymphocyte reaction was studied using a one-way mixed culture technique. In this assay γ-irradiated tumour cells were cultivated in the presence of allogeneic lymphocytes and the extent to which the lymphocytes were stimulated was determined by measuring the amount of thymidine incorporated into them.

In the first series of experiments a constant number of lymphocytes (5×10^6/ml) collected from the efferent popliteal lymph of normal sheep was used as the responder population. Various concentrations (1×10^4 - 5×10^6/ml) of γ-irradiated cultivated tumour cells were added to this population and the cultures harvested after 5 days of incubation. Stimulation was considered to have occurred if the values for thymidine uptake in the cultures containing both tumour cells and lymphocytes ±2SE (standard errors) were higher than the sum of the values of thymidine uptake obtained from cultivating each cell type alone. All cultures were set up in triplicate. Seven different tumour cell lines were tested for their ability to stimulate allogeneic lymphocytes *in vitro*. The results of a representative experiment with tumour cell line F18 are given in Table 4.1. This tumour

Table 4.1 *Inability of tumour cell line F18 to stimulate allogeneic efferent lymphocytes in vitro*

Source of stimulator	Stimulator cell concentration/ml	Thymidine uptake* cpm ± SE
-[1]	-	825 ± 165
Tumour (F18)[2]	5×10^6	397 ± 49
Tumour (F18)[3]	5×10^6	321 ± 25
"	1×10^6	529 ± 61
"	5×10^5	760 ± 95
"	1×10^5	674 ± 67
"	5×10^4	373 ± 111
"	1×10^4	182 ± 12
PBL (F18)[4]	5×10^6	87085 ±9211

1 Cultures containing responder cells only (efferent lymphocytes, 5×10^6/ml).

2 Cultures containing stimulator cells only (tumour cells, 5×10^6/ml).

3 Cultures of stimulator cells at various concentrations in the presence of responder cells (efferent lymphocytes).

4 Cultures of blood leucocytes from tumour-bearing sheep (F18) in the presence of responder cells (efferent lymphocytes).

* Cultures terminated after 120 hr of incubation. All cultures in triplicate.

failed to stimulate a response in allogeneic efferent lymphocytes. The uptake of thymidine in the mixed cultures was consistently lower than in the control. All 7 tumour cell lines tested behaved similarly in that they failed to stimulate allogeneic lymphocytes.

The inability of the tumour cells to stimulate allogeneic lymphocytes *in vitro* could have been due to a number of reasons, including compatibility between the tumour donor and the sheep from which the responder cells were obtained. This possibility was tested by setting up a mixed lymphocyte reaction (MLR) in which lymphocytes were used from the tumour-bearing sheep instead of the tumour cells. As seen in Table 4.1, significant stimulation was obtained indicating that the lack of stimulation in the tumour cell-allogeneic lymphocyte cultures was not due to histo-compatibility between the sheep from which the responding lymphocytes were obtained and the tumour donor. In all subsequent experiments, tests for compatibility between the donor tumour-bearing sheep and the sheep from which the responder cells were obtained, were carried out.

Studies by a number of investigators on the mixed lymphocyte reaction (Scollay 1972, Lafferty *et al.*, 1974) and on the tissue or tumour-lymphocyte interaction (Anderson *et al.*, 1972; Eurton *et al.*, 1975; Pawelec *et al.*, 1979) have shown that a number of variables can affect the outcome of this assay. Therefore it was necessary to test for the effect of these variables on the stimulatory capacity of the tumour cells.

In the previous experiments, the concentration of the stimulator cells (tumour cells) was varied in the assay. The effect of altering the time at which the assay was terminated, the source of responder cells and the responder cell concentration

on the outcome of the assay, was also tested.

4.2.1 *Kinetics of the Interaction*

To examine the kinetics of the interaction, cultures were set up using a constant number of responder cells (5×10^6/ml, efferent lymphocytes) and various concentrations of stimulator cells (1×10^4 - 5×10^6/ml, tumour cells). Cultures were then terminated after 3, 5 or 7 days of incubation. Fig. 4.1 shows that the tumour cell line F40 did not stimulate at any of the time periods or at any of the concentrations investigated. Thus the lack of capacity of the tumour cells to stimulate could not be changed by these manoeuvres.

4.2.2 *Variation in the Source of Responder Cells*

In the previous experiments, efferent lymphocytes were used as responder cells. It has been shown by Smith *et al.* (1970) that the efferent lymph in sheep consists mainly of small lymphocytes and is devoid of macrophages. Since it has been suggested that macrophages are required in the mixed lymphocyte reaction (Wagner *et al.*, 1972; Rode and Gordon 1974), it was decided to use peripheral blood leucocytes as responders in the assay. This was done to test whether the presence of other white cells, together with lymphocytes, had any effect on the stimulatory capacity of the the tumour cell lines.

Seven different tumour cell lines were tested for their capacity to stimulate allogeneic peripheral blood leucocytes. Table 4.2 shows the results of one representative experiment which demonstrated that tumour cell line F35 did not induce any proliferation in allogeneic blood leucocytes. This experiment

Table 4.2 *Inability of tumour cell line F35 to stimulate allogeneic peripheral blood leucocytes in vitro*

Source of stimulator	Stimulator cell concentration/ml	Thymidine uptake* cpm ± SE
-[1]	-	868 ± 95
Tumour (F35)[2]	5×10^6	233 ± 25
Tumour (F35)[3]	5×10^6	176 ± 29
"	1×10^5	265 ± 35
"	5×10^5	373 ± 19
"	1×10^5	216 ± 45
"	5×10^4	185 ± 21
PBL (F35)[4]	5×10^6	53794 ± 9729

1 Cultures containing responder cells only (PBL leucocytes, 5×10^6/ml).

2 Cultures containing stimulator cells only (tumour cells, 5×10^6/ml).

3 Cultures of stimulator cells at various concentrations in the presence of responder cells (peripheral blood leucocytes).

4 Cultures of blood leucocytes from tumour bearing sheep F35 in the presence of responder cells (peripheral blood leucocytes).

* Cultures terminated after 120 hr of incubation. All cultures in triplicate.

suggested that the presence of other white blood cells in the culture did not alter the inability of the tumour cells to stimulate allogeneic lymphocytes.

4.2.3 *Variation in the Concentration of the Responder Cells*

The final parameter which was investigated was the concentration of responder cells in the assay. Since previous data did not indicate that any specific concentration of stimulator cells was optimal, different concentrations of tumour cells were used with varying concentrations of responder cells. Fig. 4.2 shows the results of one experiment in which 3 selected doses of responder cells were used. Tumour cells again failed to stimulate a response in allogeneic leucocytes irrespective of the dose of responder cells used.

The results of the tumour cell-lymphocyte interactions *in vitro* showed that the tumour cells were unable to stimulate allogeneic lymphocytes. This inability of the tumour cells to stimulate could be a tissue culture artefact or could be related to certain properties of the cancerous cells such as (1) a high antigenic content resulting in a high zone tolerance; (2) a lack of certain alloantigens which are responsible for causing stimulation in the mixed lymphocyte reaction or (3) suppression of lymphocyte proliferation by the tumour cells. The first possibility seemed unlikely since different tumour cell concentrations were used for stimulation. The 2nd and 3rd could be tested directly and therefore experiments were designed to see whether lack of stimulation was due to a deficiency of allo-antigens on the cells or due to immunosuppressive effects on the lymphocytes.

4.3 DEMONSTRATION OF ALLOANTIGENS ON TUMOUR CELLS

Detection of alloantigens can be done either serologically or by measuring cellular reactivity directed against the respective alloantigens. Because of the time involved in the production of an alloantiserum against each respective tumour, the cellular reactivity against alloantigens was measured.

The mixed lymphocyte reaction (MLR) and a cytotoxic assay involving cell-mediated lysis (CML) are the two most extensively used *in vitro* assays for the analysis of the proliferative and effector phases of cellular immunity (Häyry and Defendi 1970). In the MLR the proliferation of the responder cells is measured. The cell-mediated cytotoxic assay analyzes the development of cytotoxic cells generated in the mixed lymphocyte cultures. Cytotoxicity is measured by the ability of the cells generated in MLR to lyse labelled target cells carrying the same antigens or other cross-reacting antigens, as the stimulator cell. Thus, the MLR and CML were used to investigate the presence of alloantigens on the tumour cells.

4.3.1 *Generation of Cytotoxic Cells in vitro*

Since it has been reported by various investigators that proliferation of cells in MLR is required for the development of cytotoxic cells (Wagner 1972; Cantor and Jandinski 1974; Hirano and Nordin 1976), experiments were set up to define the optimal conditions for cell proliferation in the MLR in the present system. Cultures were prepared with an equal number of stimulator cells (sheep peripheral blood leucocytes) and responder cells (sheep efferent lymphocytes) so that the final concentration of cells

in the culture was either 5×10^6/ml, 7×10^6/ml or 10^7/ml. Cultures were harvested on the fifth day after the cells were mixed and the extent of thymidine incorporation into the responding cell population was measured.

Results from these experiments showed that cultures containing 10^7/ml lymphocytes gave the lowest level of stimulation whereas cultures containing 5×10^6/ml or 7×10^6/ml lymphocytes gave good stimulation. The suppression of thymidine incorporation seen at the highest cell concentration (10^7/ml) may have been a non-specific effect resulting from the exhaustion of the medium at high cell densities (Kung *et al.*, 1977). In order to obtain the maximum number of cells for use in the cytotoxic assay designed to demonstrate the presence of alloantigens, a cell concentration of 7×10^6/ml was chosen for subsequent experiments.

4.3.2 *The Cytotoxicity Test*

Peripheral blood leucocytes from tumour-bearing sheep were used to stimulate efferent lymphocytes from normal sheep. Cultures were harvested at day 5 and were used as effectors against ^{51}Cr labelled tumour cell targets from the same blood donor sheep. For testing specificity, tumour cells were used from other sheep and from the xenogeneic mouse tumour P815. The results of the cytotoxic effect of cells generated in these cultures against the respective tumours are given in Table 4.3.

Cytotoxicity against tumour cells could be demonstrated in 6/8 of the tumour cell lines tested, indicating that some alloantigens were expressed on 6 of these cell lines. The cytotoxic reaction was specific since no cytotoxicity was detected against a xenogeneic mouse tumour (P815) or against other allogeneic tumour

Table 4.3 *Demonstration of the presence of alloantigens on tumour cells*

Sensitizing cell	% ^{51}Cr release* from tumours: Effector cell/target cell ratio 200:1	100:1	Unrelated cell	Effector cell/target cell ratio 200:1	100:1
F18	42	25	F6	3.0	1.6
			P815	2.5	1.2
F35	33	18	F18	-	1.2
			P815	1.5	3.0
F51	27	11	F6	15	7.6
			P815	2.3	3.9
F40	20	13	F18	-	-
			P815	2.8	4.0
F57	19	16	F6	2.2	4.2
			P815		
F58	13	8	N.T.	N.T.	N.T.
F24	3.9	-	F6	2	-
			P815	3.5	4.9
F54	3.4	2	F18	3.0	-
			P815	-	-

* 4 hr assay

\- Not detected

N.T. not tested

F24 and F54 are negative

cells. In one case, however, cross-reactivity was observed; cells sensitized against lymphocytes obtained from the F51 tumour-bearing sheep caused significant lysis of the tumour cell line F6. This cross-reactivity may have been due to the sharing of some alloantigens between the 2 sheep.

The absence of lysis in 2/8 of the tumour cell lines tested may be attributed to the absence of detectable alloantigens on these cells. Alternatively, it may have been due to decreased sensitivity of these tumour cells to immune lysis. For example, variations were observed in the total ^{51}Cr uptake, the maximum amount of ^{51}Cr released following water lysis and the spontaneous release of ^{51}Cr between the different tumour cell lines used as targets. Accordingly, since cell lines vary in their susceptibility to water lysis, there may also be variations in the resistance of different cell lines to immune lysis. Variation in susceptibility to lysis may also be a function of the number of times the tumour cell lines have been subcultured. It is known that tumour cells harvested directly from the tumour material *in vivo* and those obtained from an early subculture *in vitro* are sometimes less susceptible to immune lysis than cells that have been subcultured several times (de Vries *et al.*, 1974). In the present study, tumour cell lines were used after only a few subcultivations (2nd-6th).

The results presented so far have indicated that the inability of the tumour cell lines to stimulate allogeneic lymphocytes was not necessarily due to a general lack of allo-antigens. Moreover, it was shown clearly that sheep have cells capable of mediating cellular cytotoxicity *in vitro* and that most tumour cell lines tested can be used as targets in a cytotoxic assay. This point needed to be established prior to performing

any experiments in the autochthonous system.

4.4 SUPPRESSION OF THE MIXED LYMPHOCYTE RESPONSES (MLR) BY TUMOUR CELLS

This section investigates whether the lack of stimulation in the tumour cell-lymphocyte reaction was due to suppression of the *in vitro* immune response.

To test for possible suppressive properties of the tumour cell lines, the effect of adding tumour cells to a normal mixed lymphocyte reaction was investigated. Mixed lymphocyte cultures were set up using optimal concentrations of responder cells (efferent lymph lymphocytes, 5×10^6/ml) and stimulator cells (peripheral blood leucocytes, 5×10^6/ml) from normal sheep. Various concentrations of γ-irradiated F6 tumour cells were added to these cultures and the effect on the proliferative response in the MLR was determined. The results of this experiment are shown in Fig. 4.3.

The addition of γ-irradiated tumour cells to mixed lymphocyte cultures produced a significant reduction in thymidine uptake in the responding cells. A suppression in ^{3}H-thymidine uptake was obvious at tumour cell concentrations of 5×10^4 - 5×10^6/ml. This suppressive effect was considered unlikely to be due to overcrowding since the same number of third party allogeneic leucocytes added to the cultures did not cause any suppression of the mixed lymphocyte response. Suppression of ^{3}H-thymidine uptake occurred only in the presence of viable tumour cells since the addition of heat-killed tumour cells at high densities had no effect on the response (Table 4.4).

In total, seven tumour cell lines (F6, F18, F35, F40, F51.

Table 4.4 *The effect on mixed lymphocyte responses of adding various concentrations of dead tumour cells*

Tumour cell concentration/ml	*Thymidine uptake cpm ± SE
- †	119,662 ± 17,532
5×10^6	127,545 ± 11,479
1×10^6	136,335 ± 12,817
5×10^5	114,842 ± 10,159
1×10^5	101,379 ± 16,352

† The uptake of thymidine in mixed lymphocyte cultures without the addition of dead tumour cells.

* Cultures terminated after 120 hr of incubation.

F54 and F57) were tested for their effect on the normal mixed lymphocyte reaction and six showed the suppressive effects described above. One tumour cell line (F40) had no effect on the MLR responses (Fig. 4.4) at any of the concentrations tested. This particular tumour also lacked the ability to stimulate allogeneic lymphocytes although it could function as a target in cell-mediated lysis, indicating that the tumour expressed alloantigens.

4.5 DISCUSSION

Cultivated ovine squamous cell lines consistently failed to stimulate a response in normal sheep lymphocytes when mixed together *in vitro*. This lack of *in vitro* immunogenicity was apparent regardless of the concentration of tumour cells used for stimulation, the source of the responding cell (efferent lymphocytes or peripheral blood leucocytes) or the duration of the culture. It was also shown that the failure of the tumour cells to stimulate allogeneic lymphocytes was not due to histocompatibility between the sheep which provided the cells since mixed lymphocyte cultures between cells from the same two sheep resulted in significant responses.

The failure of the tumour cells to stimulate allogeneic lymphocytes *in vitro* does not reflect a general property of normal epithelial skin cells. It has been claimed however, by some investigators (Talmage *et al.*, 1977) that stimulation of allogeneic lymphocytes *in vitro* is dependent on the type of tissue from which the tumour derives. Thus they proposed that tumours of lymphoreticular origin stimulate allogeneic lymphocytes

in vitro, whereas tumours of epithelial origin do not. The capacity of cells to stimulate could be attributed to their expression of Ia antigens which stimulate proliferation in MLR. Ia antigens are not expressed by epithelial cells in general but only by Langerhan's cells (Rowden 1980). Since in this system the tumours used were of epithelial origin this may provide an explanation of their inability to cause stimulation in allogeneic lymphocytes. This possibility however, seems unlikely because it has been reported by Hirschberg *et al.* (1975) that cultivated normal epithelial cells, which do not express Ia antigens, were still capable of stimulating a significant response in allogeneic lymphocytes.

Another related explanation comes from the work of Lafferty *et al.* (1978) who have proposed that the allogeneic reaction occurs only when a responsive lymphocyte binds antigens through its cell-surface receptor (signal 1) and at the same time receives a stimulus from a lymphokine (signal 2). It would follow that the failure of these tumours to stimulate was due to a lack of either the binding reaction between the responder cell and the tumour cell antigen or to the failure of the tumour cell to produce a lymphokine.

An alternative explanation may be found in the fact that the majority of the tumour cell lines tested inhibited a normal mixed lymphocyte reaction and as a consequence their inability to stimulate might be a reflection of immunosuppressive capacity. It is well documented that immunosuppression can accompany tumour growth and is manifested *in vitro* by a decreased response of the lymphocytes of cancer patients to mitogens and *in vivo* by diminished delayed cutaneous hypersensitivity to a variety of

contact allergens (Stutman 1975; Kamo and Friedman, 1977). Supernatants of established cultures of human neoplasms have been shown to contain factors which can affect the non-specific lymphocyte responsiveness to mitogens (Renk *et al.*, 1980) as well as specific lymphocyte responses to tumour antigens (Dent *et al.*, 1978). It has been shown that there exists in the supernatant fluid of cultures of melanoma cell lines, certain factors which inhibit thymidine uptake by degrading the thymidine to a non-utilizable form (Dent *et al.*, 1978). The presence of such factors may explain the lack of reactivity observed when lymphocytes from melanoma patients are mixed with tumour cells.

In the present system the requirement for viable tumour cells in the culture to inhibit thymidine uptake suggests that this effect is mediated through a suppressive factor. Alternatively it may be that tumour cells cause immunosuppression by depleting the medium of a metabolite which is necessary for lymphocyte proliferation.

With respect to the tumour cell line F40, no stimulation was obtained when the tumour was cultivated with allogeneic lymphocytes. Furthermore, it was shown that lack of stimulation was not due to immunosuppression. Thus whatever mechanism was responsible for suppression of MLR by the other tumour cell lines, it was obviously not operative within this particular tumour. It is conceivable that the inability of this cell line to stimulate was due to an absence of the 2nd signal (Lafferty *et al.*, 1978).

The findings in this chapter illustrate two characteristics of the tumour cells; 1. their inability to stimulate allogeneic lymphocytes and, 2. their immunosuppressive properties on lymphocyte responses. These two characteristics could be of

importance in analyzing the host-tumour interaction in naturally occurring tumours.

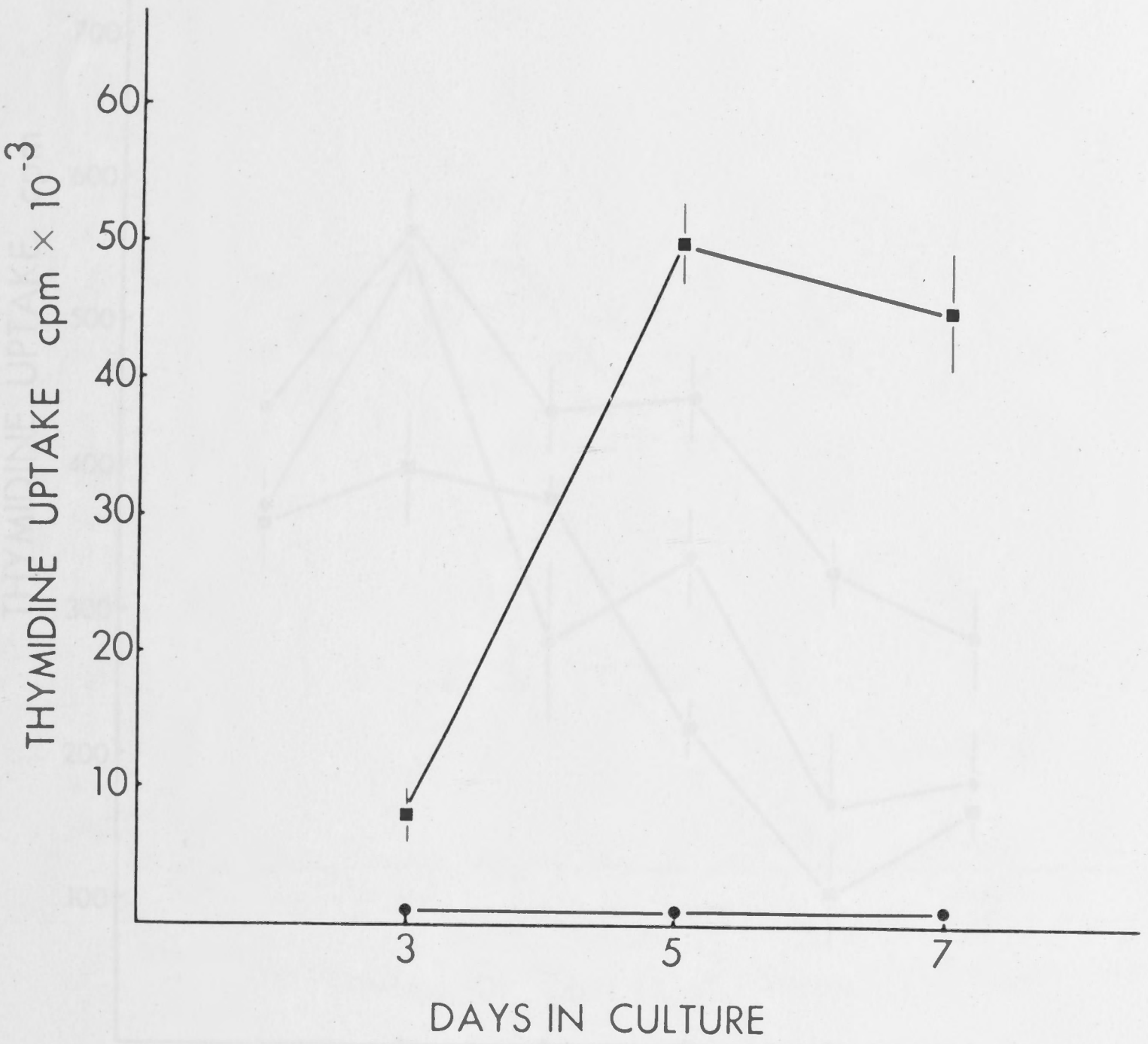


Figure 4.1 The effect of the length of the culture period on the ability of tumour cells (●—●) and peripheral blood leucocytes (■—■) to stimulate allogeneic efferent lymphocytes. Both tumour cells and lymphocytes were used at concentrations of $5X10^6$/ml.

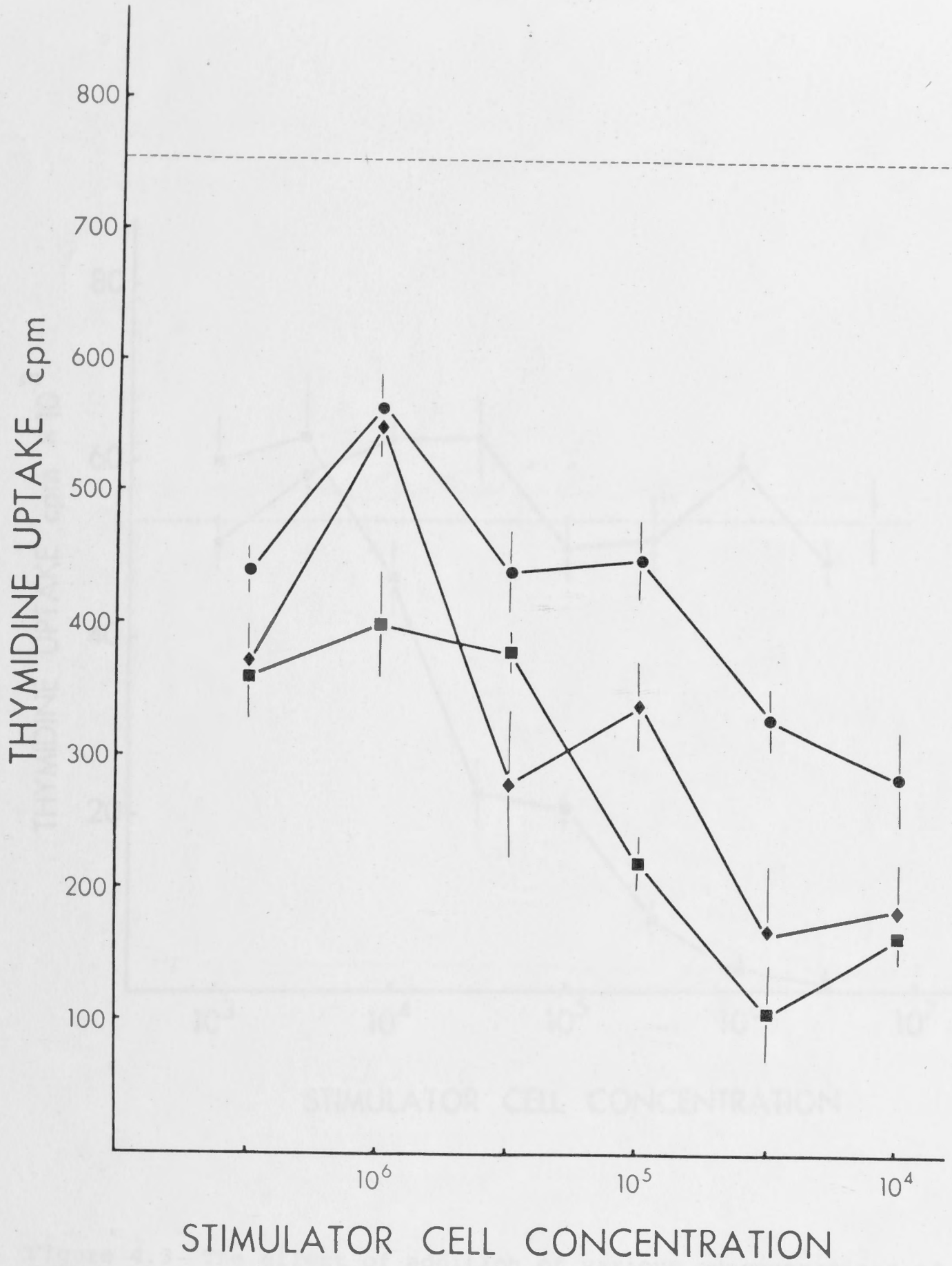


Figure 4.2 The effect of the responder cell concentration on the ability of the tumour cells to stimulate a response in allogeneic blood leucocytes. Responder cell concentrations were $5X10^6$/ml (●—●), $5X10^5$/ml (♦—♦) and $5X10^4$/ml (■—■). The dotted line represents the control which consists of the sum of the values of thymidine uptake obtained from cultures in which tumour cells and blood leucocytes were cultured alone.

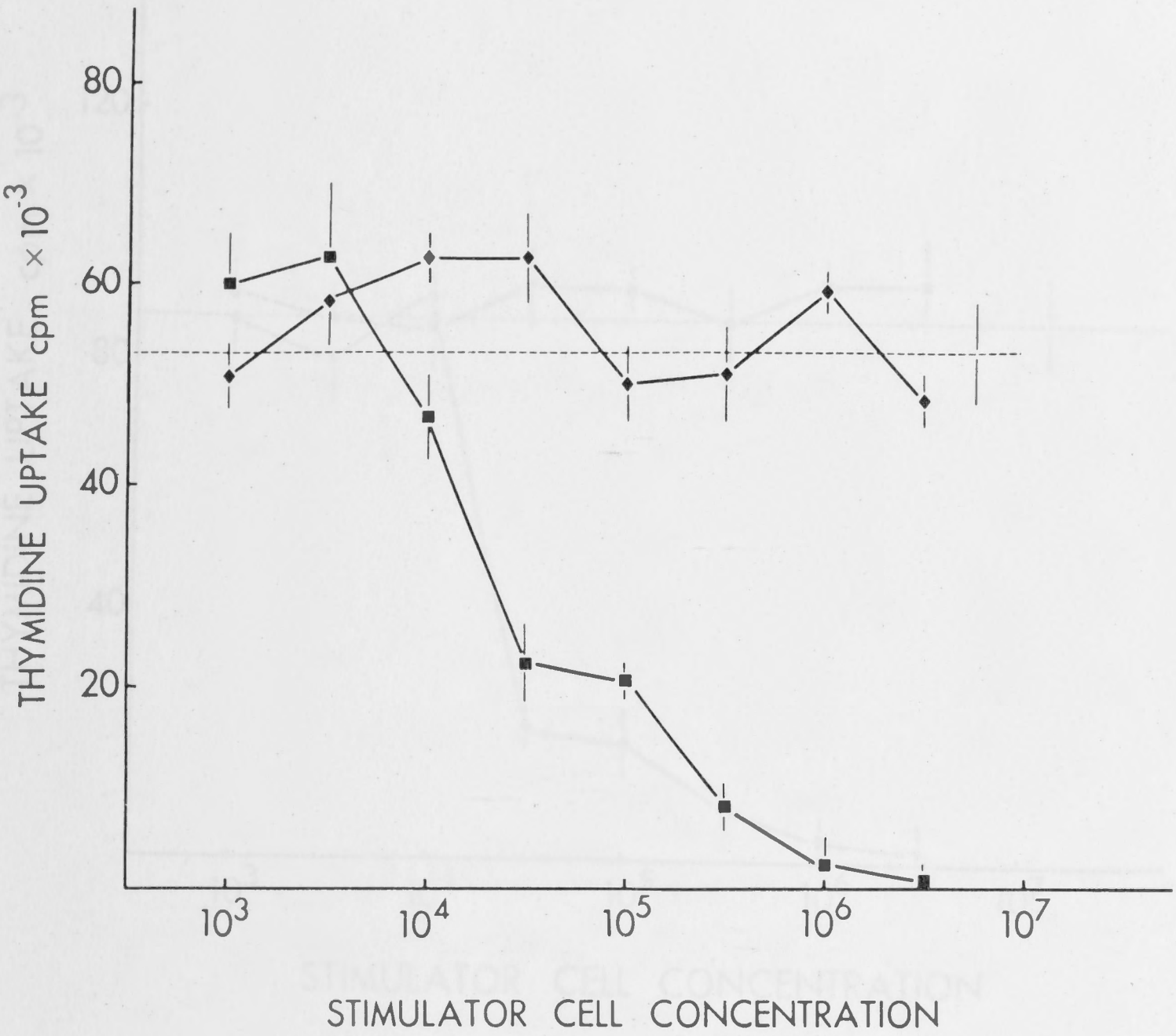


Figure 4.3 The effect of addition of various concentrations of γ-irradiated third party tumour cells (F6 (■ — ■) or peripheral blood leucocytes (♦ — ♦) on the level of stimulation obtained in a normal mixed lymphocyte reaction. The level of thymidine uptake in the mixed lymphocyte cultures without addition of third party cells is represented by the dotted line.

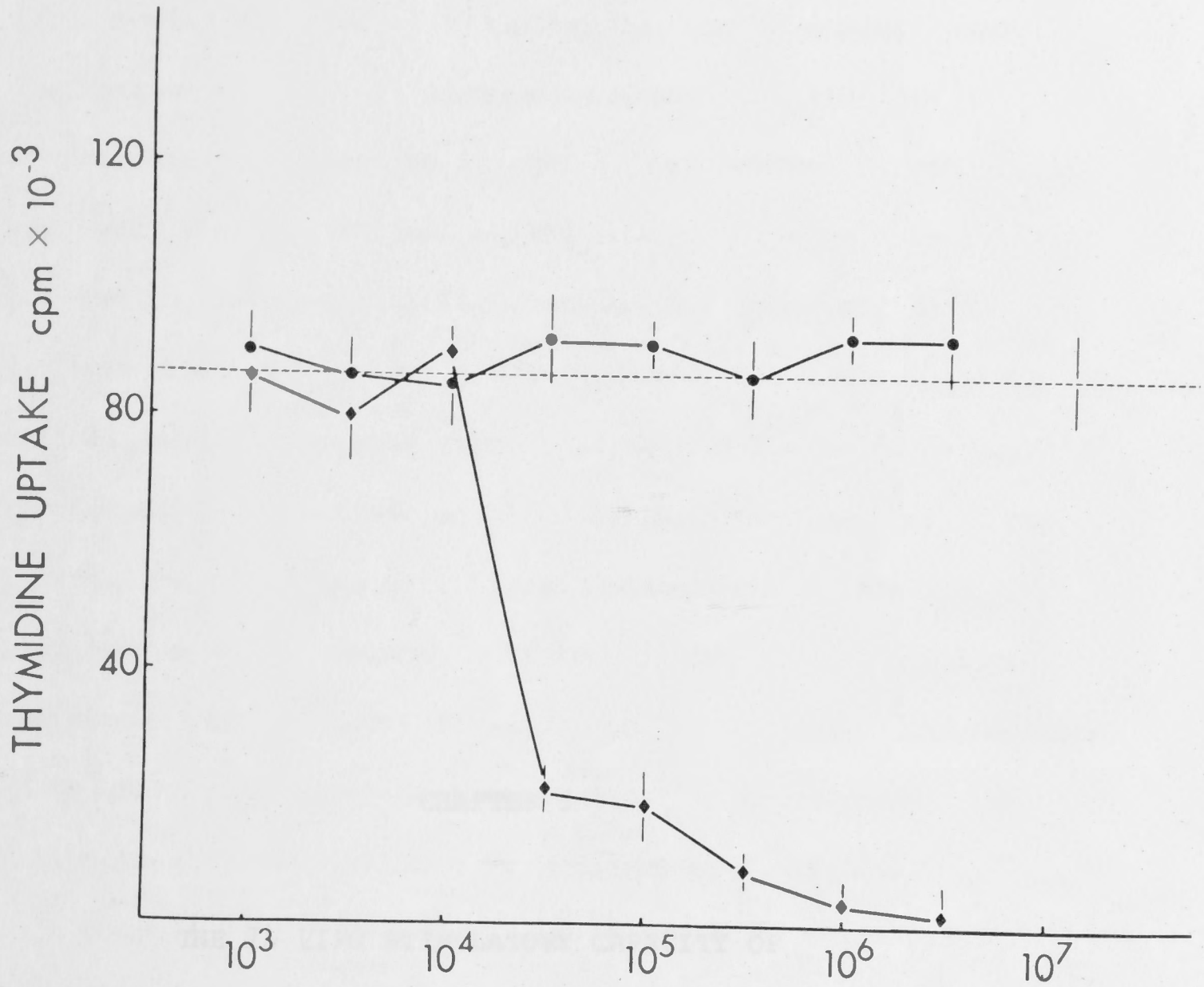


Figure 4.4 The effect of addition of various concentrations of γ-irradiated third party tumour cells F40 (●——●) or tumour cells F18 (♦——♦) on the level of stimulation obtained in a normal mixed lymphocyte reaction. The level of thymidine uptake in the mixed lymphocyte cultures without addition of third party cells is represented by the dotted line.

CHAPTER 5

THE *IN VIVO* STIMULATORY CAPACITY OF TUMOUR CELL LINES

5.1 INTRODUCTION

Results reported in the previous chapter demonstrated that the various tumour cell lines were unable to stimulate a proliferative response in allogeneic lymphocytes *in vitro*. It was shown that the failure of the tumour cells to stimulate was not due to histocompatibility between the donor and responder, nor was it due to lack of alloantigens on the tumour cells. Further studies revealed that the tumour cells had a non-specific immunosuppressive effect on mixed lymphocyte responses *in vitro*. The failure of tumour cell lines to stimulate allogeneic lymphocytes may be related to this. Studies also showed that one tumour cell line did not suppress ^{3}H-thymidine incorporation in *in vitro* mixed lymphocyte cultures. This cell line also failed to stimulate allogeneic lymphocytes *in vitro*.

Because of the contrived nature of *in vitro* systems involving cell mixtures, it was not possible to place these *in vitro* results in the context of reactions between cells *in vivo*. Experiments were therefore designed to examine the ability of selected tumour cells to stimulate a response in allogeneic sheep *in vivo*. Tumour cells were introduced into a lymph node of a sheep and the reaction that ensued was monitored in the lymph draining from the node. The experimental approach that was used is illustrated in Fig. 5.1.

Both the afferent and efferent lymphatic vessels of the popliteal lymph nodes of sheep were cannulated and 10^{7} cultivated tumour cells were infused into the node through the afferent lymphatic cannula. The immune response was confined to the

lymph node by quantitatively collecting the lymph draining from the node following antigenic challenge. In this way the temporal sequence of events that occurred in the node in response to the tumour cells could be analyzed (Hall and Morris 1965b).

The immune response was analyzed in terms of the changes in the rate of lymph flow, the total cell output and the large cell output. Both the humoral and cellular reactivities which were induced against the tumour cells were measured during the response using an *in vitro* cytotoxic assay.

Three different cell lines were selected for these experiments. These cell lines had been characterized *in vitro* with regard to their expression of alloantigens, their ability to stimulate a response in allogeneic lymphocytes and their ability to suppress a mixed lymphocyte reaction. A summary of the characteristics of these cell lines is presented in Table 5.1.

The three cell lines chosen were similar in that they all expressed alloantigens and they all failed to stimulate allogeneic lymphocytes *in vitro*. However, they differed with respect to their ability to suppress ^{3}H-thymidine uptake of cells in *in vitro* mixed lymphocyte reactions. Thus while the cell lines F6 and F18 were found to be highly suppressive *in vitro*, the other cell line (F40) was not.

Prior to carrying out the challenge experiments it was necessary to test the degree of compatibility between the tumour donor sheep and the prospective recipient of the tumour cells. This was done using a mixed lymphocyte reaction, in which peripheral blood leucocytes from the tumour-bearing sheep were used to stimulate efferent lymphocytes from the sheep designated

Table 5.1 *In vitro characteristics of tumour cell lines selected for experiments involving in vivo antigenic challenge*

Cell line number	Expression of alloantigens	Stimulation *in vitro*	Suppression of MLR
F6	+	-	+
F18	+	-	+
F40	+	-	-

\+ Yes

\- No

to receive the tumour cells. The results presented in Table 5.2 indicate that in each experiment, significant stimulation was obtained indicating that the pairs of sheep tested were histoincompatible.

5.2 RESULTS

Two different patterns of reactivity were observed following challenge with the different tumour cell lines. The reactivity obtained in response to challenge with both F6 and F18 tumour cell lines was similar and accordingly, the results from these experiments are presented together.

5.2.1 *The in vivo Stimulating Capacity of the Tumour Cell Lines F6 and F18*

The ability of the cell lines F6 and F18 to stimulate a response *in vivo* was tested in 4 sheep and 3 sheep respectively. The results from a representative experiment with each tumour cell line are shown in Fig. 5.2.

Immediately following challenge there was a sharp decline in the cell output in the lymph from the stimulated node. By day 3 following challenge, an increase in the lymph flow, the total cell output and the output of large cells was observed. At the peak of the response, the total cell output was between 30-40 $\times 10^7$ cells/hr and the output of large blast cells constituted 20-40% of the total white cells leaving the stimulated node. These changes in the efferent lymph occurred between the 3rd and 7th day following challenge with the peak of the response occurring on the 5th and 6th day. By two weeks after challenge, the lymph flow, total cell output and large

Table 5.2 *Mixed lymphocyte reactions between the tumour cell donors and the prospective tumour cell recipients*

Tumour donor[1]	Tumour recipient[2]	Thymidine uptake (cpm ± SE)
F18	FN5	56106 ± 5489
F18	FN6	27634 ± 3316
F18	FN7	38415 ± 2689
F40	FN8	114730 ±12915
F40	FN9	72923 ± 6199
F40	FN10	53221 ± 7213
F6	FN1 - FN4	N.T.

[1] Peripheral blood leucocytes from tumour-bearing sheep were used as stimulator cells in MLR.

[2] Efferent lymph lymphocytes from prospective recipients of tumour cells were used as responders in MLR; both the stimulating and the responding cell populations in MLR were used at a concentration of 5×10^6/ml.

* Cultures terminated after 120 hr of incubation. All cultures in triplicate.

N.T. = not tested, no source of peripheral blood leucocytes was available because the tumour-bearing donor sheep had died.

cell output had returned to their prechallenge levels. Although the magnitude of the blast cell response varied in the different recipients, the general pattern of events occurring in the efferent lymph was similar in all sheep challenged with the two tumour cell lines.

The specific immune response generated during challenge with the tumour cells was determined by measuring the cytotoxic effect of both efferent cells and cell-free lymph against the tumour cells used to stimulate the response.

(a) Direct cell-mediated cytotoxicity

The cytotoxic effect of efferent lymph cells against the immunizing tumour cells was tested before and at various intervals following challenge (Fig. 5.3). Low levels of cell-mediated cytotoxicity were first detected at day 4 and this activity increased gradually to reach a peak at day 7 before the activity declined. Maximal cytotoxicity was detected with an effector-target cell ratio of 200:1 and 100:1; low levels of cytotoxicity were also detected at ratios of 50:1. The cytotoxic activity displayed by efferent lymphocytes from the stimulated node was associated with the presence of the blast cells in the lymph. No cytotoxic activity was detected in the cells in the lymph before challenge or at any time after the blast cell response had disappeared.

The cytotoxic activity of the cells was specific for the immunizing tumour cells. Table 5.3 shows the result of an experiment in which a sheep was challenged with the tumour cell line F6, and the efferent lymph cells were tested for cytotoxicity against the immunizing tumour cell (F6), against another

allogeneic tumour cell (F18) and against a xenogeneic tumour cell (P815). Tests for specificity were carried out on day 5 and 6 following challenge. As shown in the table, no significant cytotoxic activity was demonstrated against the F18 tumour cells or the P815 cells on either day, whereas the cytotoxic reactivity obtained against the immunizing tumour cell F6 was 21% and 49% on day 5 and 6 respectively.

As another test for specificity of the anti-tumour cytotoxic effect of lymphoid cells produced in response to an unrelated antigen was measured. A sheep was immunized with complete Freund's Adjuvant (CFA) and at the peak of the blast cell response (16.9%), the efferent lymph cells were tested for their cytotoxicity against the tumour cell lines F6 and F18. Table 5.4 shows that the addition of lymph cells produced in response to CFA had no cytotoxic effect on the tumour cells tested, even when high effector cell to target cell ratios were examined and when long periodsof incubation were used for the assay.

The initial experiments which were done to detect cell-mediated cytotoxicity involved a 4 hr assay. However, it was found subsequently that the sensitivity of the assay could be raised by increasing the period of incubation to 24 hr. A much greater amount of the ^{51}Cr was released in assays in which the test was extended to 24 hr and this caused samples that were negative in the 4 hr test to become positive in the 24 hr test (Figure 5.4). Usually in this type of cytotoxic assay incubation for longer periods of time results in high spontaneous release of ^{51}Cr from the target cells and consequently interpretation of the significance of the data obtained is difficult. However, in the system used in the present experiments, incubation

Table 5.3 *The cytotoxic effect of efferent lymphocytes obtained from sheep immunized with the tumour cell line F6 against the immunizing tumour and against unrelated tumours*

Days after challenge with F6	% large cells in lymph	Tumour cells used in assay	*% ^{51}Cr release from tumour cells		
			Effector:target cell ratio		
			200:1	100:1	50:1
5	13.5	F6	21	11.0	5.0
		F18	-	3.4	-
		P815	-	2.0	-
6	18	F6	49	24.0	15.0
		F18	-	-	-
		P815	-	-	-

* 24 hr assay

\- Not detected

Table 5.4 *The cytotoxic activity of sheep efferent lymphocytes against tumour cells following challenge of the popliteal node with unrelated antigen#*

Cell line tested	% ^{51}Cr release† from tumour cells	
	Incubated with medium	Incubated with immune cells*
F6	26.9	29
F18	28.0	31

\# sheep were challenged with CFA; the efferent lymph contained 16.9% large cells at the time of test.

* effector cell/target cell ratio, 200:1

† 24 hr assay

was possible for 24 hr because the spontaneous release of ^{51}Cr from the tumour cell lines did not exceed 30% of the total releasable ^{51}Cr during the 24 hr period (Table 5.5).

(b) Antibody complement-dependent cytotoxicity

Complement-dependent cytotoxic antibodies were first detected in the cell-free efferent lymph on the 4th day following challenge and coincided with the appearance of blast cells in the lymph. The level of cytotoxic antibodies increased to reach a peak titre of 1/625 around the 7th - 8th day of the response. The antibody titre in the lymph declined only marginally after the blast cell response had disappeared from the lymph and the titre persisted at a high level for at least 15 days after challenge (Table 5.6). It was certain that in these experiments a significant amount of antibody was produced by cells which remained in the lymph node and never appeared in the lymph as free-floating cells. In these experiments no cytotoxicity was detected in the efferent lymph against P815 or another allogeneic tumour cell line.

5.2.2 *The Stimulatory Capacity of the Tumour Cell Line F40*

The ability of F40 tumour cells to stimulate an allogeneic response *in vivo* was next investigated. Tests for histo-compatibility between the tumour cell donor and the prospective tumour cell recipients were carried out using mixed lymphocyte reactions. These results are presented in Table 5.2.

The popliteal lymph nodes of 3 sheep were challenged with F40 tumour cells; the results of one of these experiments showing the total and large cell output in the efferent lymph at various intervals following challenge are presented in Fig. 5.5. The

Table 5.5 *Spontaneous ^{51}Cr release by tumour cells following different periods of incubation*

Target used	% Spontaneous ^{51}Cr release from tumour cells		
	4 hr	8 hr	24 hr
F6	8.9	11	27
F18	9.7	14	29

^{51}Cr released following water lysis of F6 = 8467 ± 596 = 100%.

^{51}Cr released following water lysis of F18 = 6381 ± 163 = 100%.

Table 5.6 *Cytotoxic antibodies detected in the efferent lymph following challenge with the tumour cell line F6*

Days after challenge	% large cells in lymph	*% specific ^{51}Cr release from the tumour cells in the presence of lymph at various dilutions			
		1/5	1/25	1/125	1/625
1	4	-	-	-	-
3	5	17	10	-	-
4	15	80	80	15	-
6	20	76	82	75	15
7	17	75	74	71	63
8	13	85	96	83	44
10	9	80	93	82	16
11	5	81	79	72	11
15	3	76	65	42	9

* 4 hr assay

\- Not detected

magnitude of the response obtained following challenge with F40 cells was significantly lower than the responses detected after challenge with either F6 or F18 cells, although the time course of the response was similar in all cases. The total cell output at the peak of the response ranged form 5 - 12 $\times 10^7$ cells per hr compared to 30 - 40 $\times 10^7$ cells per hr in the responses to F6 or F18 tumour cells. At the peak of the total cell output, only one of the 3 sheep showed a significant increase in the percentage of large cells in the lymph (8%). In the other two sheep challenged with this tumour cell line (F40) the percentage of large cells in the lymph reached 4% and 5% respectively.

(a) Cytotoxic mechanisms detected in the lymph following challenge with the tumour cell line F40

The cytotoxic activity of efferent lymphocytes against the immunizing F40 tumour cells was measured at various intervals following challenge. No cellular cytotoxicity was detected following challenge at any of the effector to target cell ratios tested using either a 4 hr or 24 hr incubation period. Low levels of complement-dependent cytotoxic antibodies were detected in only one of 3 sheep tested.

5.3 DISCUSSION

The response of the popliteal lymph node of sheep to a wide variety of antigens has been well documented (Morris 1968). A number of characteristic changes occur in the efferent lymph when the node is stimulated antigenically. These changes may include an increase in the rate of lymph flow, and an increase in both the total cell output and in the percentage of large

cells in the lymph. These changes in the efferent lymph occur regardless of the nature of the antigen although the magnitude and the duration (timing) of the response may vary with different antigens. Thus Hall (1964) found that the percentage of blast cells appearing in the lymph following an antigenic challenge with 6×10^8 chicken red cells reached a peak of 9% whereas in the response to 5×10^7 human red cells the percentage of the blast cells in the lymph at the peak of the response reached 12.5% (Hall *et al.*, 1967). Yamashita *et al.* (1970) showed that in responses to boiled *Salmonella muenchen* organisms the blast cells in the lymph often reached 50% of the total cells on the 3rd-4th day following challenge while Smith and Morris (1970) found that the response to 10,000 haemagglutinating units of Swine influenza virus lasted up to 10 days with the blast cells reaching a peak of 26% at day 6. Immune responses to live allogeneic lymphocytes are very vigorous and protracted. Grant and Cameron (1975) found that although the magnitude of the blast cell response in the popliteal lymph was similar for both allogeneic or xenogeneic cells, the response to xenogeneic cells occurred earlier; changes in the efferent lymph were detected 3-4 days following challenge with xenogeneic cells whereas those detected in response to challenge with allogeneic cells occurred 2 days later.

In the present experiments significant cellular responses were detected in the efferent lymph following challenge with 2 of the 3 tumour cell lines tested. The blast cell responses were associated with the detection of both cellular and humoral cytotoxic reactivities directed against the immunizing tumour cells. The cytotoxic activity of the lymphocytes in the efferent

lymph was specific for the immunizing cell and was detected only during the blast cell response. From the results it seemed clear that it was the transformed, activated lymphocytes that were responsible for the reaction. No cytotoxicity against the tumour cells was observed following incubation of the tumour cells with blast cells obtained from animals immunized with unrelated antigens or with other tumour cells.

While the mechanism of the cytotoxic reaction and the precise nature of the cytotoxic cell in the efferent lymph are not known the cytotoxic reaction could be either antibody dependent or be mediated directly by cells. If the reaction was antibody dependent, then it could be mediated either by "K" cells, representing an antibody-dependent cellular cytotoxicity (ADCC) mechanism of killing, or it could be complement dependent (Grant and Cameron, 1975). The ADCC is unlikely to be the mechanism whereby the tumour cells were killed in the present system for two reasons. First, it has been shown by other investigators (Grant *et al.*, 1975) that cells capable of mediating ADCC in the sheep (K-cells) are not present in the efferent lymph although they are found in the blood and have characteristics in common with those of blood monocytes. Secondly, I have shown that efferent lymphocytes obtained from sheep immunized with one particular tumour cell line (F18) were incapable of mediating cytotoxicity against another tumour cell line in the presence of antibodies specific for the latter (data not shown).

It has been suggested by other investigators (Grant *et al.*, 1970, 1971) that the blast cells in the efferent lymph of sheep can synthesize complement. Accordingly Grant *et al.* (1970, 1971) attributed the cytotoxic reaction observed during the blast cell

response to an antibody complement-dependent mechanism. This explanation would seem unlikely, because if blast cells can synthesize and release complement, it would be expected that cytotoxicity would be detected when these cells were added to cultures containing antibodies specific for the target cells.

The cytotoxic reaction could be cellular in nature and mediated by either NK cells, macrophages or T cells. NK cells are excluded from being the effector cells in this sytem because by definition, these cells do not require previous sensitization to mediate their killing effect and no cellular cytotoxicity was detected in the efferent lymph before challenge.

The possibility that macrophages could be the mediators of the cytotoxicity either in a specific or non-specific sense also seems unlikely as it has been shown by other investigators (Smith *et al.*, 1970) that the efferent lymph of the sheep is devoid of macrophages. There are, however, some circumstances in which macrophages may enter efferent lymph following antigenic challenge although their appearance is usually evanescent.

It is possible that the cytotoxic reaction was mediated by T-cells similar to those cells which are present in mice. Although T-cells in sheep can be detected by certain surface markers, no isolation procedures for T-cells were done in the present experiments and consequently it is difficult to come to definite conclusions about the nature of the cytotoxic cell.

Direct cell-mediated cytotoxicity which could be attributed to T-cells has been reported in the sheep by Grant and Cameron (1975). More recently Pearson *et al.* (1979) reported a T-cell-mediated cytotoxic mechanism detected in cattle infected with the *Theileria parva* parasite. The T-cell nature of the cytotoxic

cells was confirmed by a rosetting technique and by the absence of surface immunoglobulin on the cells (Emery, Personal Comm. 1981).

The results presented here raise the question as to why the tumours F6 and F18, which failed to stimulate an allogeneic response *in vitro*, can stimulate *in vivo*. Lack of correlation between the results obtained *in vitro* and *in vivo* has been reported by many investigators. Baldwin and Embleton (1974) found that lymphoid cells from rats with spontaneous or chemically induced tumours were sensitized against the tumour cells when tested *in vitro*. These tumours were unable to elicit significant tumour transplantation resistance *in vivo* which suggests that the responses detected *in vitro* do not contribute significantly to host immunity. Moreover Ilfeld *et al.* (1973) found that murine lymphoid cells sensitized *in vitro* against either fibroblasts or syngeneic tumour cells (3LL Lewis tumour) were equally cytotoxic against 3LL tumour target cells. However, *in vivo*, the lymphoid cells sensitized against syngeneic fibroblasts promoted tumour growth. In the present experiments two tumour cell lines did not behave *in vivo* as would be expected in terms of the theory of allogeneic reactivity proposed by Lafferty *et al.* (1978). Their ability to stimulate *in vivo* could not be attributed to contamination of the tumour cell lines with stimulator cells of lymphoid origin because the tumours had been propagated in culture for at least 3 generations before they were used for challenge and the cell lines contained no cells of lymphoid origin. It seems most likely that histocompatibility antigens associated with the tumour cells were responsible for the immune response and this response was strong and vigorous by any

standards. It is obvious that if a suppressive factor was responsible for the lack of stimulation obtained *in vitro*, then this factor did not manifest its activity *in vivo*. Possibly the suppressive factor became diluted *in vivo* where it had no effect on the large number of lymphocytes leaving the stimulated node. These results stress the importance of conducting both *in vitro* and *in vivo* studies together before any definite conclusions can be made about the relevance of an *in vitro* phenomenon to real life.

The reason behind the observed difference between the stimulatory capacity of F6, F18 and F40 is not known. However there are many factors that could affect the capacity of cells to stimulate other cells and consequently it is not surprising that the various tumour cell lines exhibited variable reactivities when injected into allogeneic recipients. One important factor which seems to influence the stimulatory capacity of cells is their metabolic activity which may relate to the rate of turnover of specific proteins in the cell membrane (Wagner 1973). Alternatively, the physical properties of the different tumour cells may also affect their ability to stimulate. It may be that the tumour cell lines differ in their susceptibility to attack by allogeneic macrophages. Since the processing and presentation of antigen by macrophages is necessary for the stimulation of lymphocytes (Brunda and Raffel 1977), differences in the stimulatory capacity of the different cell lines may be reflected in differences occurring in the tumour cell-macrophage interaction. However, evidence is lacking as to what extent any of these factors contribute to the development of an immune response *in vivo*.

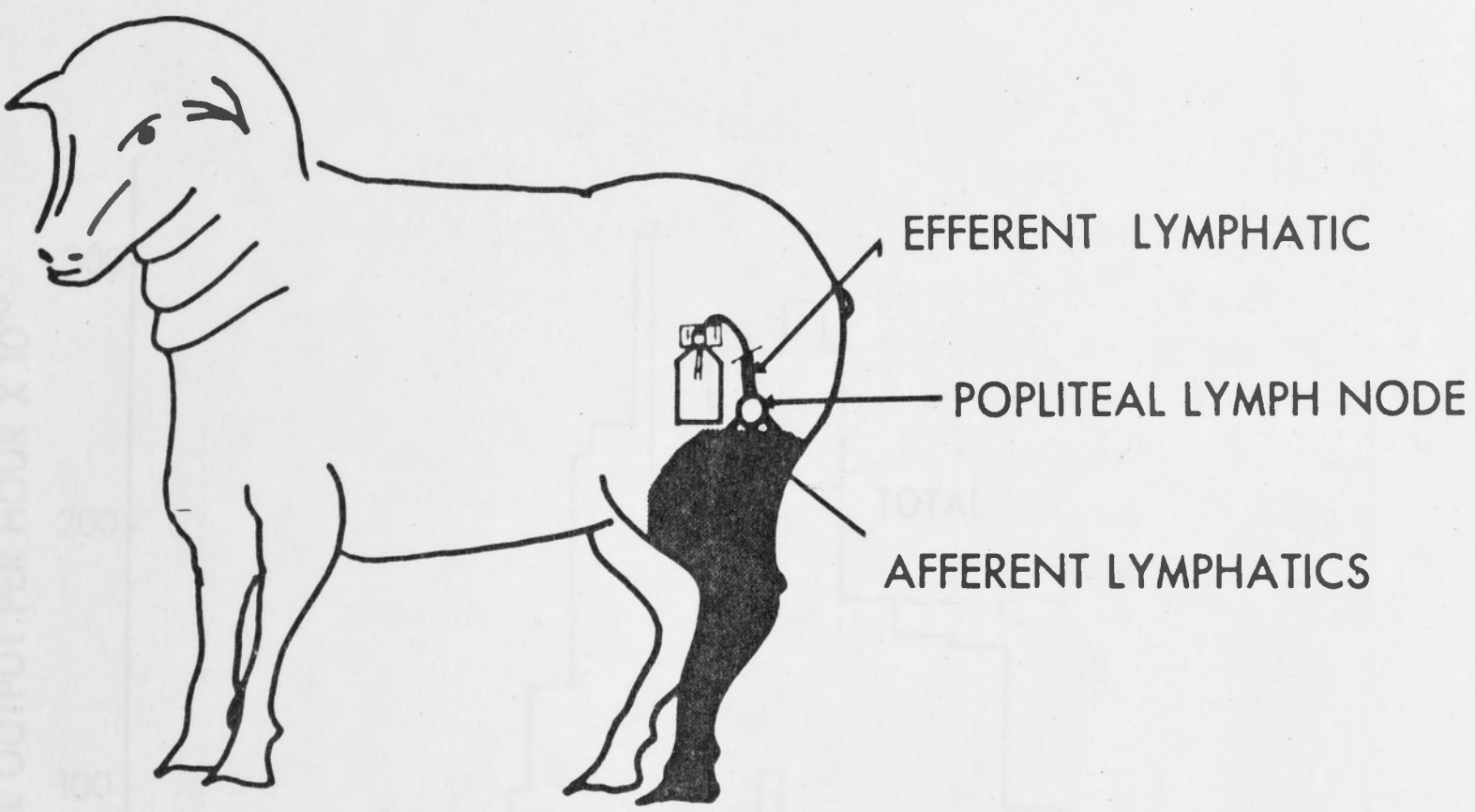


Figure 5.1 Experimental protocol for studying *in vivo* reactions to tumour cells. The afferent and efferent lymphatics of sheep were canulated and the sheep challenged with allogeneic tumour cells introduced via the afferent lymphatic. The subsequent responses in the popliteal lymph nodes were followed by monitoring changes in the efferent lymph.

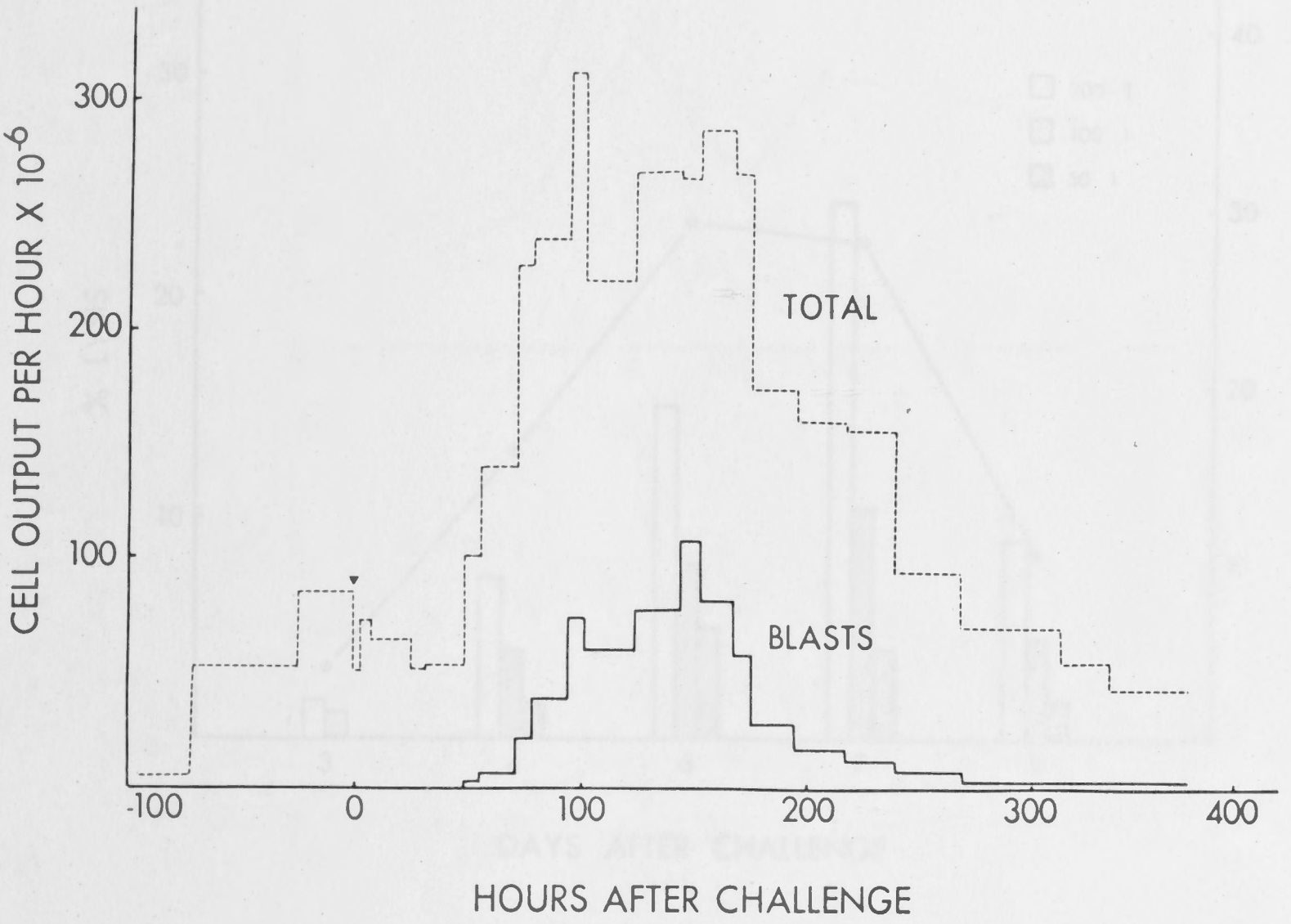


Figure 5.2 The cellular events occurring in the efferent popliteal lymph following challenge of the popliteal lymph node with 10^7 cultivated tumour cells (F6).

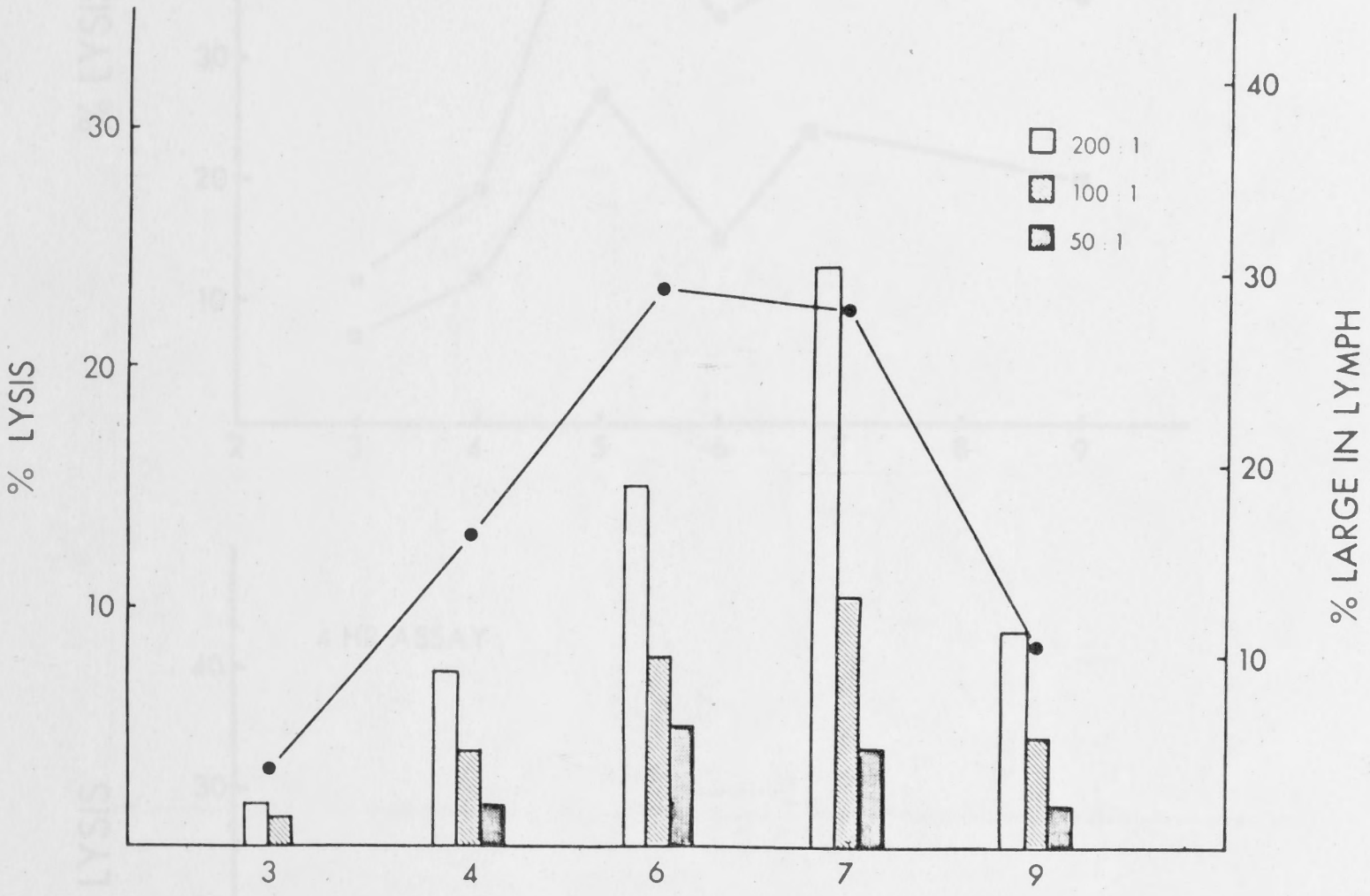


Figure 5.3 Direct cell-mediated cytotoxicity detected in the efferent popliteal lymph following challenge of the popliteal lymph node with allogeneic tumour cells at effector/target cell ratios of 200:1, 100:1 and 50:1 (4 hr assay). The percentage of blast cells detected in the total cell population in the lymph at each time interval is also presented (● —— ●).

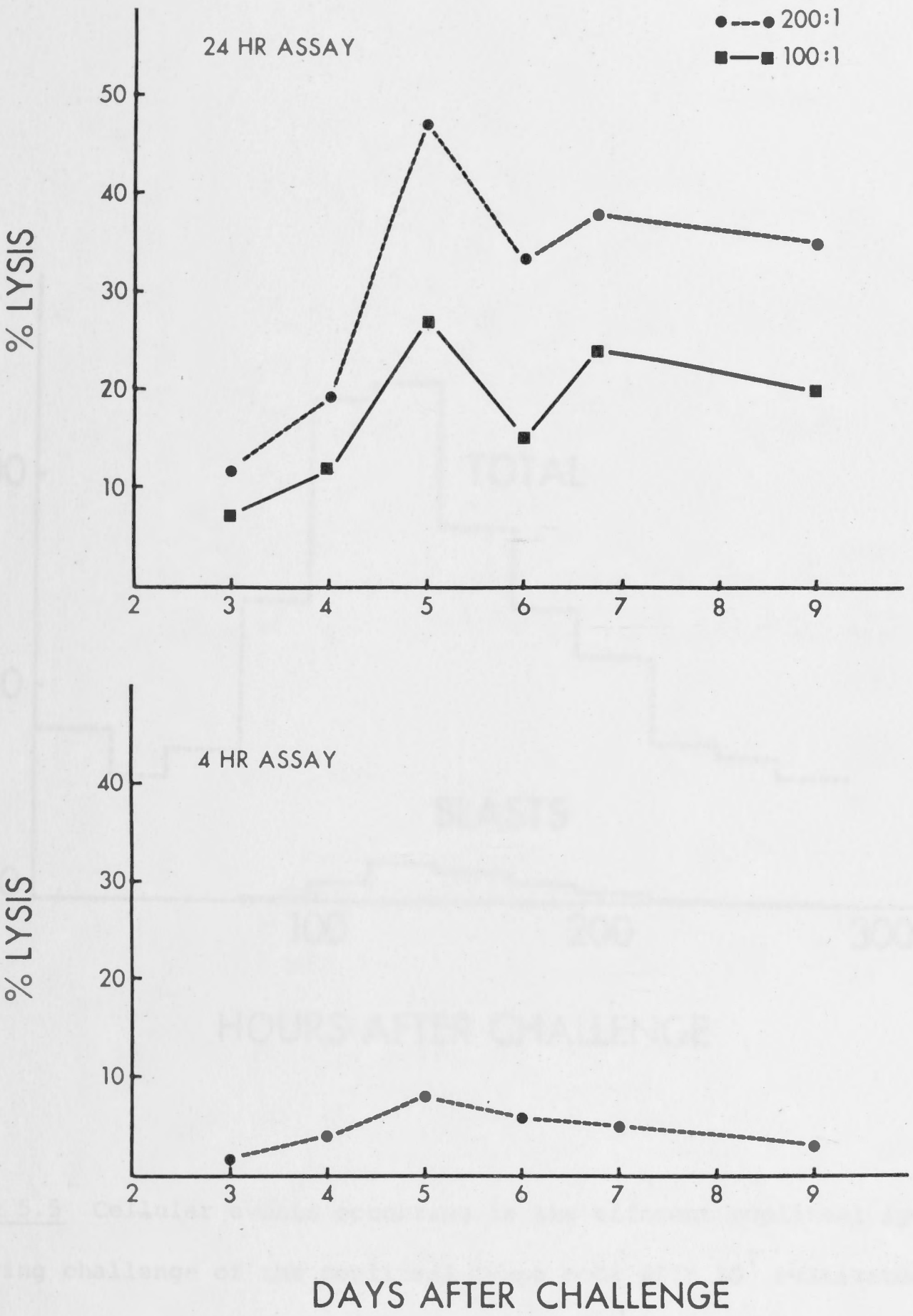


Figure 5.4 Direct cell-mediated cytotoxicity detected in the efferent popliteal lymph following challenge of the popliteal lymph node with tumour cells (24 hr assay). Effector cell/ target cell ratio 200:1 (●---●) and 100:1 (■——■).

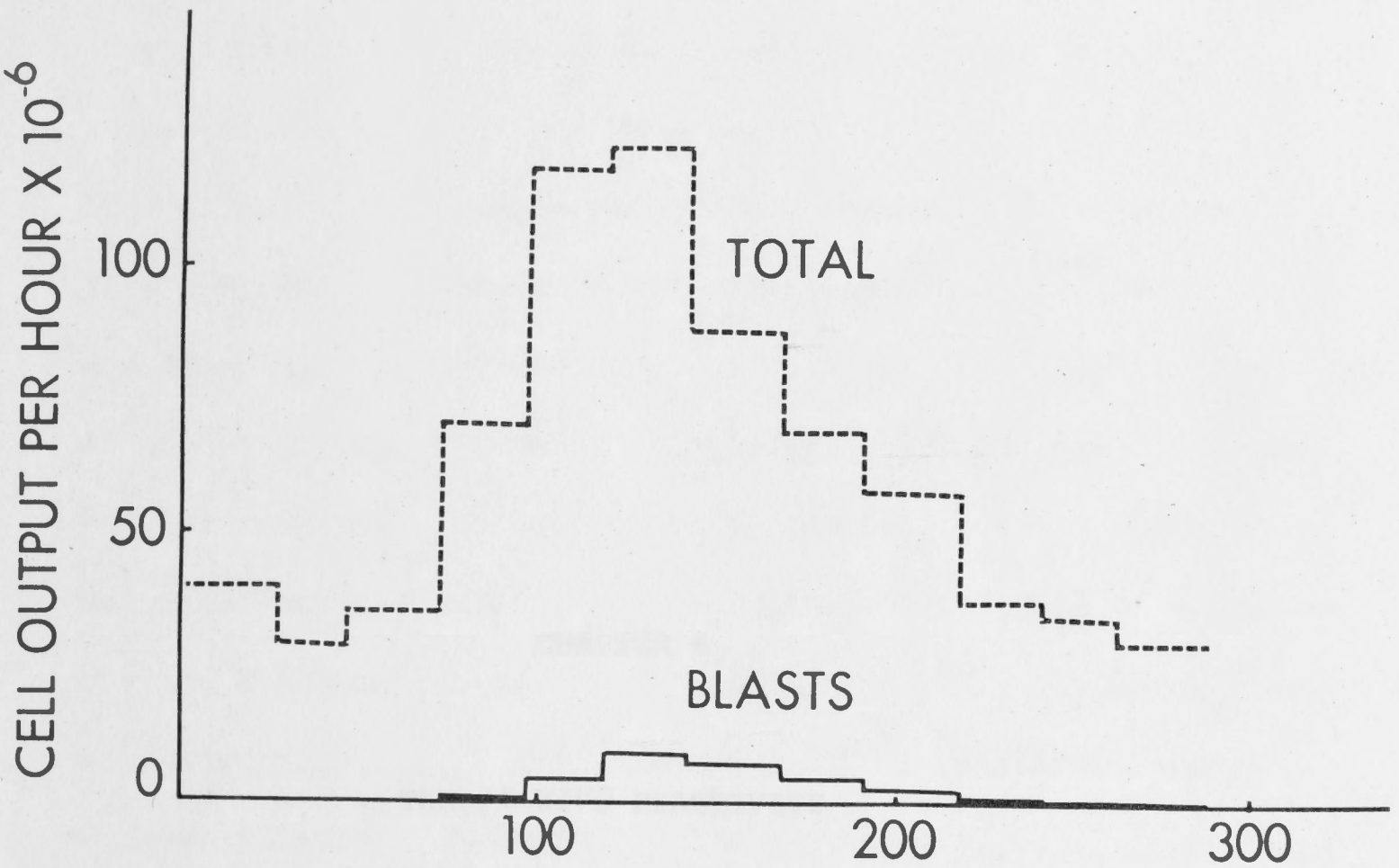


Figure 5.5 Cellular events occurring in the efferent popliteal lymph following challenge of the popliteal lymph node with 10^7 cultivated tumour cells (F40).

CHAPTER 6

THE *IN VIVO* REACTIVITY AGAINST AUTOCHTHONOUS TUMOUR EXPLANTS AND CELL LINES

6.1 INTRODUCTION

There are very few investigations into autochthonous tumour immunity in experimental animals when compared to the number of studies done in syngeneic animals and even fewer investigations with spontaneously-arising tumours in an autochthonous system. The main reason for this would seem to be that it is usually more expedient and easier to do experiments in a syngeneic system where multiple factors can be tested simultaneously in a large group of animals. Despite the advantages of studying tumour immunity in syngeneic systems, these studies are not comparable to investigating the reactions to tumours in autochthonous situations. For example, the response of an animal bearing a growing tumour of an unknown age, to a further challenge with that tumour will undoubtedly be different to the response of a naive animal to challenge with a syngeneic tumour. Since the question of whether a host can mount an immune response against its autochthonous tumour cells is the major question of relevance to the human cancer problem, the study of autochthonous tumour systems is of capital significance.

The first direct evidence for the existence of autochthonous anti-tumour immunity in an experimental system was reported by Klein *et al.* (1960). An immune resistance was demonstrated against methylcholanthrene (MCS)-induced sarcoma in mice whose autochthonous tumours were reimplanted following surgical removal of the primary tumour, and a period of inoculation with irradiated tumour cells. Mikulska *et al.* (1966) have shown that reimplantations of autochthonous benzopyrene-induced fibrosarcoma cells were rejected in the original host if the size of the primary

tumour was very small or if the primary tumour was resected. If the primary tumour was large and was left *in situ*, the tumour cells were accepted. Mikulska *et al.* (1966) failed to demonstrate any reactive lymphocytes in the spleens of animals with large tumours; specific anti-tumour reactivities were detected however, in spleen cells 3 weeks after the tumour was removed. In another study, using the same tumour-animal model, Alexander and Hall (1970) provided further evidence for the existence of tumour immunity in tumour-bearing animals after removal of the primary tumour.

The existence of tumour specific immunity to spontaneously-arising tumours is not yet accepted by many workers in the field of tumour immunology (Hewitt *et al.*, 1976, Hewitt 1978). Nevertheless, Morton (1962) reported that the growth of the first transplant generation of a spontaneous mammary carcinoma in C3H/HEN mice conferred a significant degree of immunity on the animals to subsequent transplants of the same tumour. Morton's studies were later confirmed by Riggs and Pilch (1964) who were able to demonstrate anti-tumour immunity in the primary host following removal of the primary tumour. The extent of the immunity was related to the time interval between the removal of the primary tumour and the introduction of the secondary tumour implant.

Experiments described in this Chapter were aimed at investigating whether an animal with a growing tumour can respond to a challenge with autochthonous tumour cells. The first approach was to implant fresh biopsy material to a site on the body distant from the primary tumour, observe the growth of the tumour implant and then monitor the immune response in the lymph coming from the regional node draining the tumour implant.

Subsequent experiments were done using cultivated tumour cells as challenge material. These cells were infused directly into the lymph node via an afferent lymphatic, and the immune response was again monitored in the efferent lymph.

6.2 RESULTS

6.2.1 Challenge with Tumour Autografts

Early attempts were made to establish the growth of tumours in the autochthonous host by implanting tumour tissue into the lower part of the hind leg. This area was chosen because of the relative ease with which the efferent lymphatic of the lymph node draining that area (popliteal lymph node) can be cannulated. The tumour tissue was implanted 2 days after the popliteal efferent lymph duct was cannulated as described in Chapter 2.

Eight tumour-bearing sheep were grafted with tissue fragments taken from their own tumour. Table 6.1 shows the location and size of the primary tumours from which the tumour grafts were taken. As seen in the table, no growth was observed with any of the tumour grafts. Moreover, no detectable cellular changes were observed in the efferent lymph of the popliteal lymph node draining the site of the tumour implant (Table 6.2).

There are several possible explanations as to why the tumour transplants failed to grow. One reason could be that lack of growth of the tumour was due to immunological rejection by the host. This possibility, however seems unlikely since none of the sheep studied demonstrated any changes in the efferent lymph from the regional node indicative of an immune response. This finding does not preclude the possibility that the destruction of the

Table 6.1 *The fate of autochthonous tumours transplanted into the lower part of the hind legs of sheep*

Sheep number	Tumour site	Tumour size (cm)	Site of implant	Growth * observed
F 3	nose	6 x 5	lower part of hind leg	none
F22	"	3 x 3	" " "	none
F 5	ear	7 x 6	" " "	none
F14	"	8 x 9	" " "	none
F 4	vulva	4 x 5	" " "	none
F 9	"	12 x 9	" " "	none
F21	"	11 x 14	" " "	none
F23	"	8 x 7	" " "	none

* 2 - 4 week observation period

Table 6.2 *Cellular events in the efferent lymph of the popliteal lymph nodes of sheep following the implantation of autochthonous tumour grafts into the lower part of the leg*

Days after challenge	Flow rate ml/hr	TWC/ml	LC/ml	TWC/hr	LC/hr	% large
0	3.75	4.96	.07	18.6	.26	1.4
1	3.3	6.5	.09	22.0	.30	1.4
2	2.9	4.5	.07	13.1	.20	1.5
3	4.2	4.0	.08	16.8	.34	2.0
4	5.2	3.9	.10	20.3	.52	2.6
5	4.7	5.3	.11	24.9	.52	2.1
6	4.5	5.8	.11	26.1	.50	1.9
7	4.3	6.4	.09	27.5	.39	1.4
8	3.9	5.4	.12	21.1	.47	2.2
9	3.4	4.9	.11	16.7	.37	2.2
10	4.3	4.2	.11	18.1	.47	2.4
12	3.4	3.5	.07	11.9	.24	2.0
14	3.2	4.2	.08	13.4	.26	1.9

TWC/ml total white cell concentration per ml ($\times 10^{-6}$)

LC/ml large cell concentration per ml ($\times 10^{-6}$)

TWC/hr total white cell output per hour ($\times 10^{-6}$)

LC/hr large cell output per hour ($\times 10^{-6}$)

tumour graft may occur within the graft itself without causing any detectable changes in the draining lymph node. Alternatively the failure of the tumours to grow could be related to the size of the tumour transplant. Since a tissue graft was used, the relative amounts of normal and tumour material in the graft was not known. It was possible therefore, that the transplant did not contain sufficient tumour material to initiate and/or support tumour growth. The failure of the grafts to grow could also have been related to the site of transplantation. It has been shown by several investigators that the ability of an animal to reject or accept a tumour autograft is dependent in part upon the site into which the tumour is implanted (Howard 1963; Haskill *et al.*, 1976).

To test the possibility that there are preferred sites for the growth of this particular epithelial tumour, attempts were made to graft similar sized pieces of autochthonous tumour to various sites on the body. Pieces of tumour tissue were grafted subcutaneously into the shoulder, the dorsal chest region, the flank and the lower part of the hind leg. The results of these experiments are presented in Table 6.3, which shows the location and the size of the primary tumour and the site to which pieces of the primary tumour were grafted.

The implantation of tumour tissue subcutaneously into several different locations resulted in growth. All grafts placed in the shoulder region (3/3), in the dorsal chest region (3/3) or in the flank (4/4), grew. Growth was observed in only one graft (see Tables 6.1 and 6.3), of a total of 12 grafts placed in the lower part of the hind leg. Since all tumour grafts transplanted at different sites were similar in size, the results suggested

Table 6.3 *The fate of autochthonous tumours transplanted into the subcutaneous tissue at different sites in the sheep*

Sheep number	Tumour site	Tumour size (cm)	Site of implant	*Growth observed
F18	nose	14 x 12	lower part of hind leg	No
			flank region	Yes
			dorsal chest region	Yes
			the shoulder	Yes
F19	vulva	6 x 6	lower part of hind leg	Yes
			flank region	Yes
			dorsal chest region	Yes
			the shoulder	Yes
F24	vulva	8 x 6	lower part of hind leg	No
			flank region	Yes
			dorsal chest region	Yes
			the shoulder	Yes
F37	ear	8 x 9	lower part of hind leg	No
			flank region	Yes

* 2-4 week period of observation

that the failure of the tumour grafts to grow in the lower part of the hind leg reported in the early experiments was unlikely to have been due to the grafts being of an insufficient size. Moreover, these observations indicated that there were preferential sites for tumour growth to occur.

The data presented above demonstrated that tumour grafts can be grown when transplanted subcutaneously into the flank. This area is drained by the prefemoral lymph node. Subsequently, experiments were carried out using the flank as a site for transplanting tumour grafts and for measuring any immune response which occurred in the efferent lymph of the prefemoral node draining the transplant site.

The efferent lymphatics of the prefemoral lymph nodes were cannulated in two tumour-bearing sheep. Two days after cannulation, each sheep was given an autograft of tumour tissue subcutaneously into the flank and the efferent lymph was then monitored for the next 2 weeks. Although the tumour implants grew during the first 10 days after implantation, no detectable cellular changes were observed in the efferent lymph (Table 6.4).

The failure of the animal to respond to its own tumour could be attributed to a number of factors. One obvious factor was the influence of the presence of the primary tumour. It has been demonstrated by a number of investigators (Gershon *et al.*, 1967; Barski and Youn 1969; Vaage 1971; Deckers *et al.*, 1973) that tumour-bearing animals are able to react to and subsequently reject a secondary implant of the same tumour. This condition in which a tumour continues to grow progressively despite an immune response that prevents a graft of the same tumour from growing elsewhere in the body is termed concomitant immunity. Concomitant immunity

Table 6.4 *The cellular events in the efferent lymph from the prefemoral lymph node following implantation of an autochthonous tumour graft in the drainage area*

Days after challenge	Flow rate ml/hr	TWC/ml	LC/ml	TWC/hr	LC/hr	% large
0	4.1	9.6	.28	39.4	1.20	2.7
1	4.0	9.4	.23	37.8	.92	2.4
2	4.2	9.5	.19	39.9	.78	1.95
3	4.1	8.9	.17	36.5	.70	1.9
4	4.0	7.3	.13	29.2	.52	1.8
5	3.9	8.2	.12	32.0	.47	1.5
6	3.8	9.8	.18	37.2	.68	1.8
7	3.7	8.0	.15	29.6	.56	1.9
8	3.4	10.5	.24	35.7	.82	2.3
9	3.9	7.9	.16	30.8	.62	2.0
10	3.4	8.6	.25	29.2	.85	2.9
12	2.9	6.4	.17	18.6	.49	2.6
14	1.7	6.2	.15	10.6	.26	2.5

TWC/ml total white cell concentration per ml ($\times 10^{-6}$)

LC/ml large cell concentration per ml ($\times 10^{-6}$)

TWC/hr total white cell output per hour ($\times 10^{-6}$)

LC/hr large cell output per hour ($\times 10^{-6}$)

declines as the primary tumour increases in size and this reduction in anti-tumour immunity coincides with a reduction in the general non-specific responsiveness of a tumour-bearing animal's lymphocytes. Both specific and non-specific immune reactivities are restored when the primary tumour is removed (Whitney *et al.*, 1974). The period that elapses before the immune reactivity of the host is restored varies depending on the tumour system studied. Since all the sheep I have studied had primary tumours of an unknown age, it is possible that the presence of the primary tumour may have accounted for the lack of responsiveness observed in these animals. To test this possibility, the effect of removal of the primary tumour on a subsequent challenge with autochthonous tumour cells was investigated. It was not possible to do these experiments by autografting pieces of tumour tissue because once the primary tumour was removed, no tumour tissue was available for subsequent autografting. The approach chosen therefore was to biopsy the tumour, grow it in culture as a pure population of epithelial cells and use these cells for challenge. This method had the advantage that tumour material was available for challenging the host at any time. Moreover, quantitative studies in which a precise number of tumour cells were used could be carried out.

6.2.2 *Challenge with Cultivated Tumour Cells Before Removal of the Primary Tumour*

The sheep chosen for these experiments, with one exception, were those with primary tumours on their ears. These sheep were chosen because tumours at this site are easily removed by surgery. The tumour challenge was made by infusing tumour cells into the popliteal lymph node as described previously in Chapter 5 and

specific anti-tumour cellular and humoral reactivities were determined in the efferent lymph using the established cytotoxic assay.

Prior to challenge, peripheral blood and efferent lymph were tested for both cellular and antibody-mediated cytotoxicity directed against the tumour. None of the animals tested had any detectable cytotoxic reactivity. Animals were then challenged with 10^7 cultured autochthonous tumour cells and the changes in the efferent lymph were monitored. The results of a representative experiment are presented in Figure 6.1.

The total white cell output before challenge was 5×10^6 cells per hr. Immediately following challenge there was a sharp decline in the total cell output so that by 4 hr, the total cell output had dropped to 1×10^6 cells per hr. This fall in the total cell output was transient and was followed by a steady increase so that by 24 hr following challenge the total white cell output was higher than the prechallenge level. Further changes in the efferent lymph which consisted of an increase in the total cell output and large cell output were observed by day 5, with the peak of the response occurring at day 6-7. At this stage the total cell output was approximately 60×10^6 cells per hr and the blast cell response constituted 13% of the total. Although the extent of the cellular changes in the lymph was less (between $30\text{-}60 \times 10^6$ cells/hr) than that which occurred in responses to allogeneic tumour cells ($300\text{-}400 \times 10^6$ cells/hr), the percentage of blast cells in the lymph was high. The changes described above occurred in 5 out of 6 sheep studied, with the only differences observed being in the timing and magnitude of the response. For example, the blast cell response ranged between

11% - 27% of the total cell output among the different sheep tested.

In the remaining sheep (F40), an increase in the total cell output was observed following challenge with tumour cells and a peak of approximately 45×10^6 cells per hour was reached at day 6 - 7 (Fig. 6.2). This was followed by a gradual decline in the cell output from day 8 after challenge. Although the increase in the total cell output was similar to that observed in other sheep tested, there was no significant increase in the large cell output. Moreover the characteristic fall in the cellular output which occurred immediately following challenge in the other sheep was not observed in this animal.

Both cellular and antibody-mediated complement-dependent cytotoxicity against the tumour cells were investigated in the lymph. No detectable cellular or humoral cytotoxic reactivities were found in any of the sheep tested. Since it has been demonstrated by other investigators (Denham *et al.*, 1969, Grant and Cameron 1975) that the blast cell response was always accompanied by the appearance of both cellular and humoral cytotoxic reactivities, I was surprised that I did not detect any positive cytotoxic response. The question arose as to the significance of the observed blast cell response. It was possible that it may represent a type of non-cytotoxic response which was not detected by the cytotoxic assay, and that antibodies were being produced. Experiments were carried out to investigate whether any of the blast cells appearing in the lymph during the response were producing immunoglobulin, and whether any tumour-specific antibodies can be detected in the efferent lymph. The presence of Ig-positive cells was determined by an indirect

immunoperoxidase method and detection of tumour specific antibodies was done with an ^{125}I binding assay.

Approximately 20 - 25% of the blast cells were found to be Ig positive. This percentage was similar to that obtained with the blast cells produced in responses to allogeneic tumours (Fig. 6.3). The presence of antibodies specific for the tumour in the lymph was confirmed using the binding assay. Results from these experiments demonstrated clearly that antibodies which were specific for the tumour were produced in the response. These antibodies were first detected in lymph at day 5, and increased to a titre of 1/16 at day 8. They then declined as the blast cell response declined but were still detectable in the lymph at the end of the experiment (Fig. 6.4). The appearance of these antibodies in the lymph was associated closely with the appearance of the blast cell response.

6.2.3 *Challenge with Cultivated Tumour Cells after Removal of the Primary Tumour*

The effect of removing the primary tumour on the immune response of the host to a subsequent challenge with tumour cells was investigated in a series of experiments in tumour-bearing sheep which had had a previous challenge with tumour cells in the popliteal node. The primary tumour was resected as completely as possible and the popliteal lymph node which received the first challenge was also removed. This was done to remove any tumour material that might be present in the node from the previous inoculation. Three weeks following the removal of the tumour and the challenged node, the popliteal lymph node on the contralateral side was cannulated and challenged with tumour cells as described previously (Chapter 5). The changes in the efferent lymph were

monitored and the results of a representative experiment are presented in figure 6.5.

Three weeks after removal of the primary tumour and just prior to the secondary challenge, peripheral blood and efferent lymph were tested for any cellular or humoral cytotoxic reactivities against the immunizing tumour. None of the animals tested showed any cytotoxic responses either in the blood or in the lymph. In general, similar changes to those observed in the primary challenge occurred in the lymph following the secondary challenge. These included mainly an increase in the total cell output and the large cell output. However, the secondary challenge was accompanied by a significant blast cell response. The percentage of blast cells in the lymph was approximately 2 fold higher than that obtained with the challenge given before removal of the primary tumour. Moreover changes in the efferent lymph occurred earlier; between days 3-5 following challenge. Thus, the timing and intensity of these responses were different from those described for the primary challenge and indeed they had the characteristics of a typical secondary immune response.

(a) Cytotoxic mechanisms detected in lymph following secondary challenge

Tests were carried out throughout the experiment to investigate the production of cellular and humoral cytotoxic responses directed against the tumour. Figure 6.6 shows that significant levels of cellular cytotoxicity were first detected 5 days after challenge; this reactivity increased to a peak by day 8 and then declined. No activity was detectable by day 10. The appearance of this cytotoxic activity was associated closely with the appearance of blast cells in the lymph and it declined in parallel with the

decline of these cells.

Low but persistent levels of cytotoxic antibodies were present in the lymph during the response (Fig. 6.7). Antibodies were first detected at day 4 following challenge and they increased in titre to a peak titre of 1/16 at day 7 and persisted at that level until the end of the experiment. These cytotoxic antibodies also appeared in the lymph in parallel with the blast cell response but they persisted after these cells had left the lymph, indicating that a component of this population of antibody-forming cells was restricted to the node. The peak antibody titres ranged between 1/8 - 1/32 among the different animals tested.

(b) Specificity of the cytotoxic reaction

Both cytotoxic cells and cytotoxic antibodies were tested against a panel of 2 other allogeneic tumours and against the xenogeneic mouse tumour P815. No cytotoxic cells produced by any of the sheep tested reacted with any other tumour except the immunizing one. Table 6.5 shows the combined results of all the specificity testing experiments. As seen in the Table, high levels of cytotoxicity were always obtained with the immunizing cell, ranging between 27% -75% as compared to less than 5% cytotoxicity against the allogeneic tumours (F6 and F18) and against the xenogeneic cell line (P815).

The specificity of the antibody response was also determined. In only one instance did the cytotoxic antibodies react against cells of another allogeneic tumour (F6) apart from the immunizing cells (F35).

Table 6.5 *Test for specificity of the cytotoxic reaction of lymphocytes sensitized against autochthonous tumours*

Sheep number	% large cells in lymph	Days after challenge	% cytotoxicity*			
			Immunizing cell	P815	F6	F18
F35	27	8	75	1.4	-	-
F51	33	5	56	4.5	2.3	1.7
F54	25	5	32	5	1.4	-
F57	17	6	27	-	-	-
F58	23	7	31	4.1	2.3	4.9

* 24 hour assay at effector:target ratio of 200:1

\- not detected

6.2.4 *Specificity of the Blast Cell Response*

To determine whether the blast cell response obtained was due to organ specific skin antigens or tumour associated antigens, both popliteal lymph nodes of tumour-bearing sheep were cannulated and challenged either with cultivated tumour cells or with an equal number of cultivated epithelial cells from the tumour donor. Results obtained from this experiment are presented in Figure 6.8. A significant blast cell response was obtained following challenge with tumour cells whereas no response was detected in the side challenged with normal epithelial cells. These results indicated that the blast cell response detected following challenge with autochthonous tumour cells was a response to tumour-associated antigens rather than skin-specific antigens or histocompatibility antigens.

6.3 DISCUSSION

Autografts of tumour tissue transplanted subcutaneously into the lower part of the leg failed to grow whereas successful growth was obtained when the autografts were transplanted subcutaneously into other sites of the body such as the shoulder, the chest or the flank. These observations may be explained in terms of tumours having preferred growth sites or by the more general explanation that the lower leg may present an inappropriate graft bed due to its lack of subcutaneous tissues or to a deficient blood supply. It has been shown previously by several investigators that the ability of a tumour graft to grow is partly dependent on the site of transplantation. Haskill *et al.* (1976) described a murine

adenocarcinoma that grew progressively when injected subcutaneously into the shoulder but failed to grow when injected subcutaneously into the abdomen. In another study, Auerbach *et al.* (1978a), described an antero-posterior gradient of tumour growth along the mid dorsal line between the first vertebra and the sacrum in adult mice injected intradermally with tumour cells. Faster tumour growth was obtained when tumours were injected in the anterior part of mice than in the posterior part. This difference between the anterior and posterior injection sites was also manifest after a subcutaneous inoculation (Auerbach *et al.*, 1978a) and was observed with a wide variety of different types of tumours. The differences in tumour growth between different inoculation sites was independent of the sex of the host, or the level of immunocompetence and was not related to the immunogenicity of the tumour (Vaage 1973; Auerbach *et al.*, 1978b). Studies on auto-transplanted human tumours of different histologic types are also in agreement with the above data. Howard (1963) reported that the muscular tissue provided a better "soil" for tumour growth than did the subcutaneous tissue. Six of the 22 tumour autografts transplanted into muscular tissues grew compared with 1 of 30 transplanted into subcutaneous tissues. The reasons why tumour transplants grow in certain sites and not in others may be related to the vascularity of the transplantation site, as sites supporting the growth of tumour transplants have in general a more extensive vascular bed than those where tumours grow less readily. Studies by Auerbach *et al.* (1978a) suggested that tumour growth may be regulated by factors which control growth and differentiation during embryogenesis. They proposed that such factors may still be operative in the adult, and produce the

variations in tumour growth seen when tumours were transplanted to different anatomical locations on the host.

Although tumours grew when transplanted subcutaneously into the flank, they produced no detectable changes indicative of an immune response in the efferent lymph of the prefemoral lymph node draining the site where the tumour was growing. The introduction of cultivated tumour cells into the lymph node however, resulted in a significant increase in the number of blast cells in the efferent lymph draining the challenged node. The difference between the host response to challenge with cultivated tumour cells and a tumour autograft could be attributed to a number of factors. First the route of immunization may play a role. The cultivated tumour cells were infused directly into the node, and in this situation the tumour cells would be more accessible to the host's immune system than an autograft placed some distance from the node. Secondly, the presence of lymphoid cells and/or antibodies within the graft itself may interfere with the sensitization of the host. Thus antigenic sites on the tumour cells could be masked by antigen-antibody complexes at the graft site which would prevent the sensitization of the draining node. Failure to detect reactivity in sheep challenged with tumour autografts may of course reflect a true unresponsiveness (tolerance) of the animal to its own tumour. Thus the reactivity detected in sheep challenged with cultivated cells may be due to changes in the antigenic structure of these cells induced by tissue culture. These changes may have acted to break the tolerant state and enable the animal to respond to its own tumour. It is well known that tolerance to an antigen can be broken by the introduction of the same antigen altered slightly by chemical means (Parish 1971),

by heat aggregation (Azare 1966) or by other treatments.

Following challenge with cultivated tumour cells, a significant increase in the output of blast cells occurred in the efferent lymph from the challenged node. This blast cell response was not accompanied by any cytotoxic response, either cellular or humoral, that could be detected *in vitro*. However, the response was accompanied by low levels of antibodies that bound to the tumour cells without causing their demise. The failure to detect any cytotoxic immune mechanism *in vitro* which might be of protective value to the host was attributed to the presence of the primary tumour which interfered with the host's immunity. After the primary tumour was removed and the sheep rechallenged with tumour cells, a secondary type blast cell response occurred in the lymph from the challenged node. This blast cell response was accompanied by both cellular and antibody complement-dependent anti-tumour cytotoxic activity. The blast cell response was directed specifically against tumour antigens, since no response was obtained when normal epithelial cells from the same tumour donor sheep were used instead of tumour cells. The cellular cytotoxic reactions detected *in vitro* following challenge with autochthonous tumour cells were also specific for the immunizing tumour. No cellular cytotoxicity was detected against allogeneic tumours or against the xenogeneic tumour P815. These data supported the proposition that individually specific tumour antigens existed on the tumour cells. An alternative explanation would be that the tumours possess cross-reacting antigens which were not revealed because of the restriction placed on the cytotoxic reaction by the histocompatibility complex (MHC). By way of example, sheep A would recognize tumour antigens in association with MHC type A and

lymphocytes from this animal would not kill tumour cells having similar tumour antigens if they were associated with a different MHC type. There is a histocompatibility requirement between the effector and target cell for cytotoxic T-cell reactivity to be detected (Zinkernagel and Doherty 1974; Shearer *et al.*, 1975). This feature of the T cell responses has been described recently in humans (Dickmeiss *et al.*, 1977; Goulmy *et al.*, 1977) and possibly in cattle (Emery, personal comm. 1981).

The absence of any detectable cellular immune reactivity in the host whilst the primary tumour persisted could be either a non-specific phenomenon due to general debilitation of the immune system or a specific depression of immune responsiveness towards the tumour antigens. Non-specific depression of cell-mediated immunity is frequently demonstrated in patients with cancers of various histologic types. In particular many patients suffering from squamous cell carcinoma of the head and neck have been shown to have a major impairment of their cellular immunity as measured by unresponsiveness to antigens such as 2.4-dinitrochlorobenzene (DNCB) and by a decreased responsiveness of their blood lymphocytes to T-cell mitogens (Catalona *et al.*, 1973; Eilber *et al.*, 1974; Lundy *et al.*, 1974; Wanebo *et al.*, 1975). Similarly, studies on sheep with squamous cell tumours have revealed a general non-specific decrease in cell-mediated immunity. This was manifest by a decrease in responsiveness of the blood lymphocytes of tumour-bearing sheep to phytohaemagglutinin (PHA). The level of decreased responsiveness was related to the size of the primary tumour (Jun *et al.*, 1979). A specific decrease in the responsiveness of blood lymphocytes from tumour-bearing sheep to tumour cell extracts has been described by Jun *et al.* (1979) and the extent

of the depression correlated with the size of the primary tumour. A specific decrease in host responsiveness to tumour antigens has also been described in various other tumour systems (Stjernswärd 1968; Vaage 1971).

There is considerable evidence which shows that tumour antigens, both free and complexed with antibodies, are capable of blocking cell-mediated cytotoxic reactions. The first indication that this phenomenon existed came from the observation that sera taken from tumour-bearing animals can prevent immune lymphocytes from killing tumour cells carrying the same tumour antigens (Hellström *et al.*, 1969). After this first description of blocking antibodies, similar effects were found to occur in a variety of experimental tumour systems, both spontaneous and induced. These blocking effects, detected *in vitro*, showed some correlation with tumour growth *in vivo* (Hellström and Hellström 1974) and there is some evidence that the reactivity of lymphoid cells from tumour-bearing animals may be inhibited by serum factors which become bound to their surface. These blocking factors were found to be both antigen-antibody complexes and tumour antigens (Baldwin and Price 1976a, 1976b).

There is direct evidence that both circulating tumour antigens and antigen-antibody complexes are present in tumour-bearing animals. In rats inoculated with syngeneic tumour cells, Baldwin and Price (1976a) were able to detect tumour antigens in the circulation 7 days following inoculation and circulating antigen-antibody complexes after 11 days. The presence of circulating tumour antigens and antigen-antibody complexes have been also demonstrated in humans with tumours (Heimer and Klein 1976, Samayoa *et al.*, 1977).

The mechanism by which either tumour antigens or antigen-antibody complexes depress or eliminate cell-mediated cytotoxicity is not known. However there are several possibilities. Tumour antigens either alone or complexed with antibodies could cause the observed anergy by binding to and neutralizing the effector cells, leaving fewer cells to react against the tumour cells (Vaage 1971, Baldwin and Price 1976b). Alternatively, tumour cells can suppress cell-mediated immune responses directly through the production of suppressive factors (Jun and Johnson 1979b).

Jun and Johnson (1979b) have shown that squamous cell carcinomata in sheep produce a heat-stable factor which can inhibit the response of blood lymphocytes of tumour-bearing sheep to mitogens and to the tumour extracts. The nature of such a factor is unknown but it has been shown that this factor increases in concentration as the tumour increases in size and disappears within 5 weeks following removal of the primary tumour. The disappearance of the factor coincides with recovery of cell-mediated immunity in the tumour-bearing sheep. Similarly, evidence for the existence of suppressive mechanism(s) associated with the sheep tumour was provided in Chapter 4.

The detection of a reactivity in sheep which had the characteristics of a secondary immune response, and which was apparent following removal of the primary tumour and rechallenge with tumour cells, indicated that the sheep were sensitized to tumour antigens. Failure to detect such reactivity before removal of the primary tumour could have been due to suppressive effects of the tumour on cell-mediated immunity. The inability to detect any cytotoxic mechanisms in tumour-bearing sheep before a secondary cell challenge could be attributed to incomplete recovery of these

animals from the suppressive effect of the primary tumour at the time the test was done. Jun and Johnson (1979a) reported that complete recovery of cell-mediated immunity of sheep following removal of the primary tumour takes 5 weeks. In the present experiments the first tests were carried out three weeks after the tumours were removed. In the absence of any evidence for the presence of blocking factors in the tumour-bearing sheep, suppression of cellular immunity directly through the production of suppressive factors seems the most likely explanation of the immunological defect.

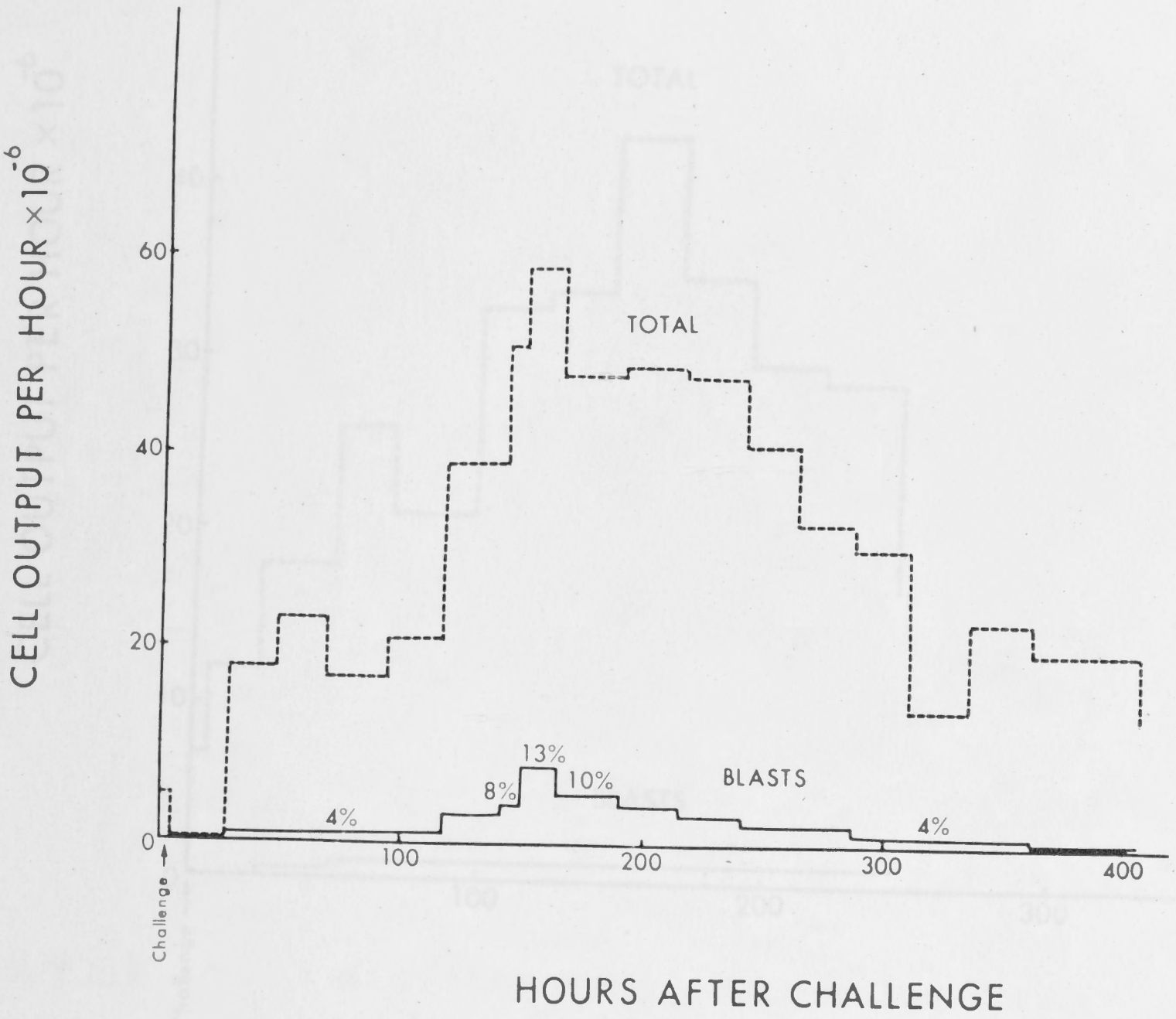


Figure 6.1 The cellular events occurring in the efferent lymph of the popliteal lymph node following challenge with autochthonous tumour cells in the presence of the primary tumour (F35).

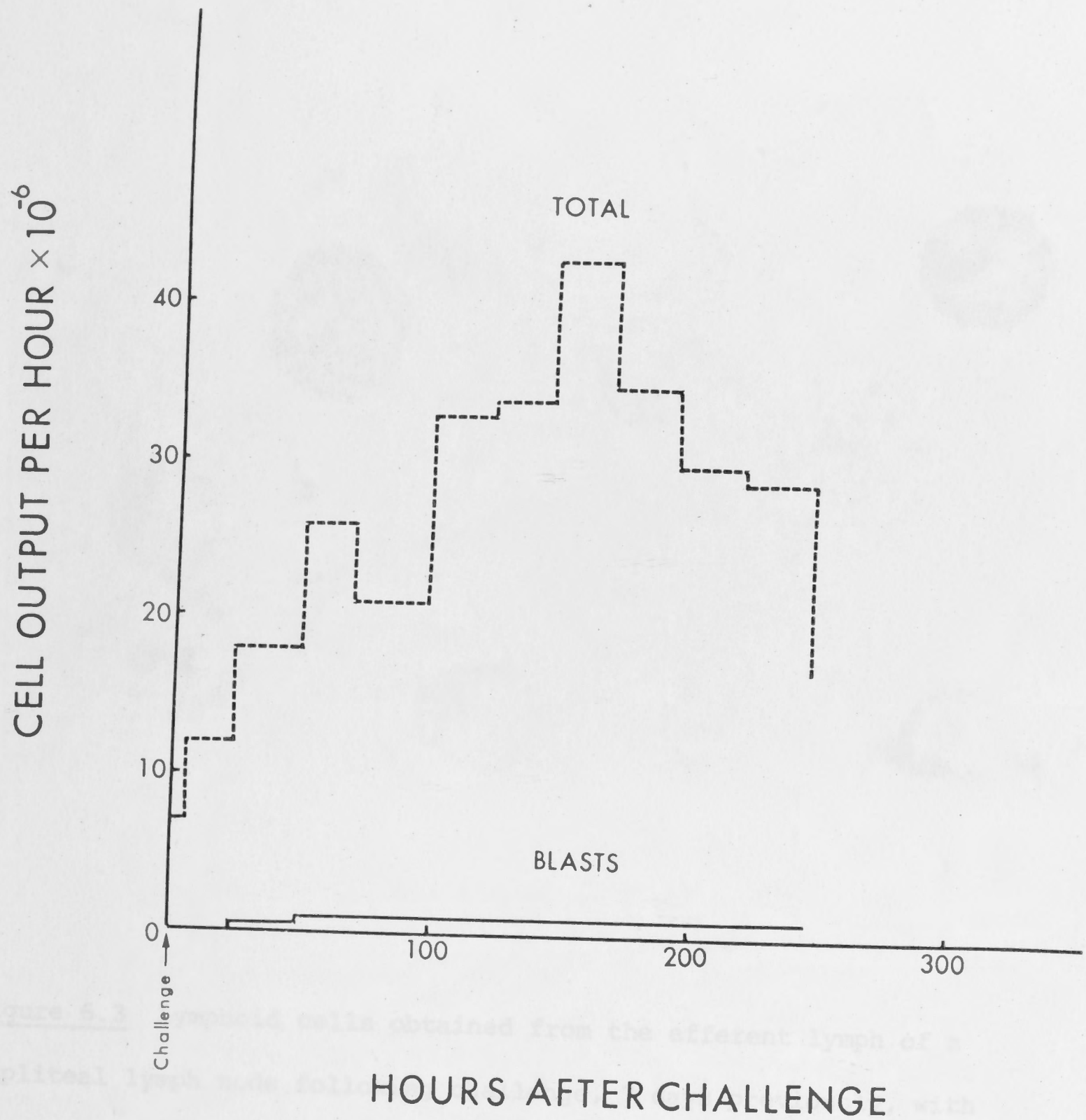


Figure 6.2 The cellular events occurring in the efferent lymph of the popliteal lymph node following challenge with autochthonous tumour cells in the presence of the primary tumour (F40).

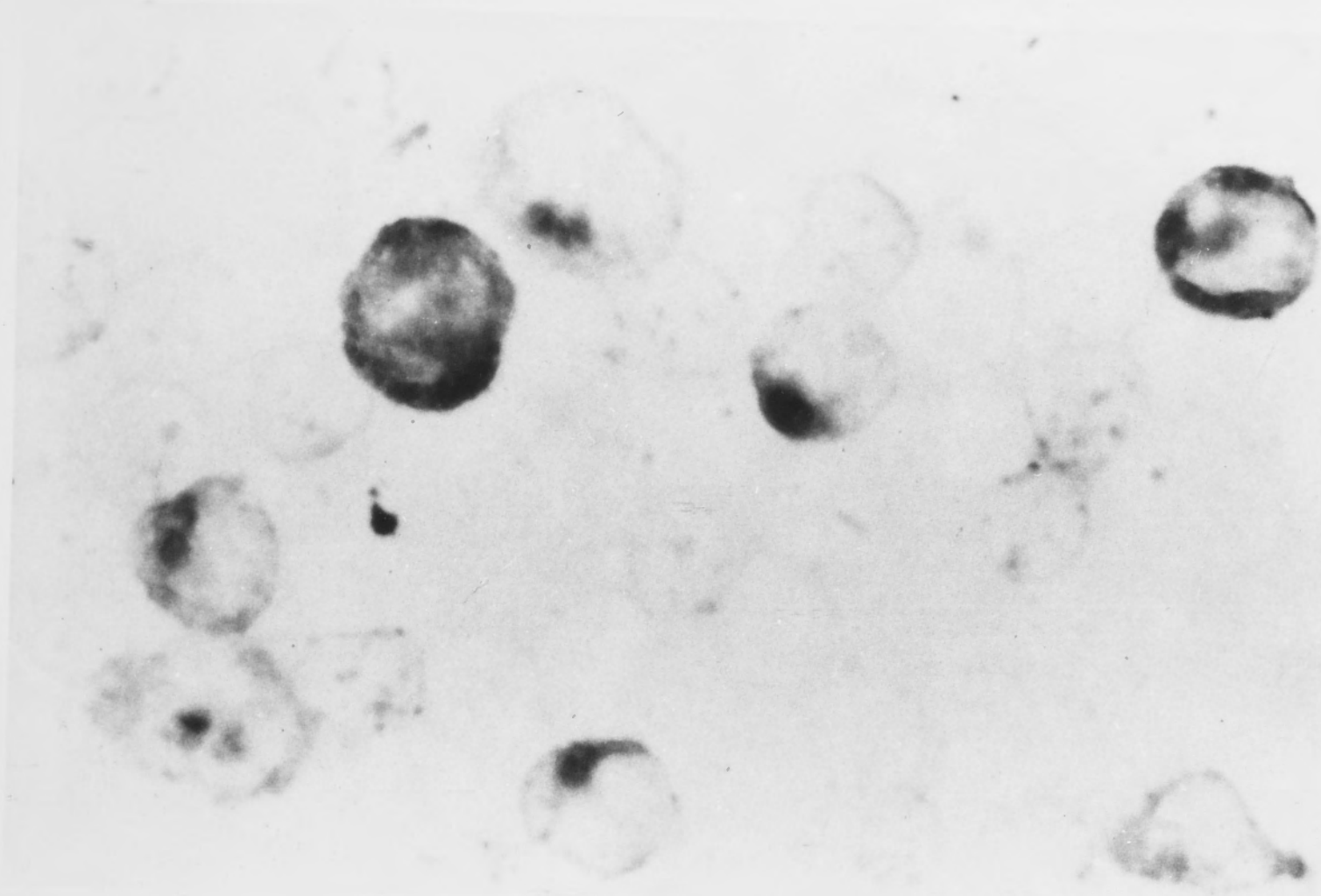


Figure 6.3 Lymphoid cells obtained from the efferent lymph of a popliteal lymph node following challenge, 7 days previously, with autochthonous tumour cells; 20-25% of the blast cells stained positive for immunoglobulin (Magnification X312.5).

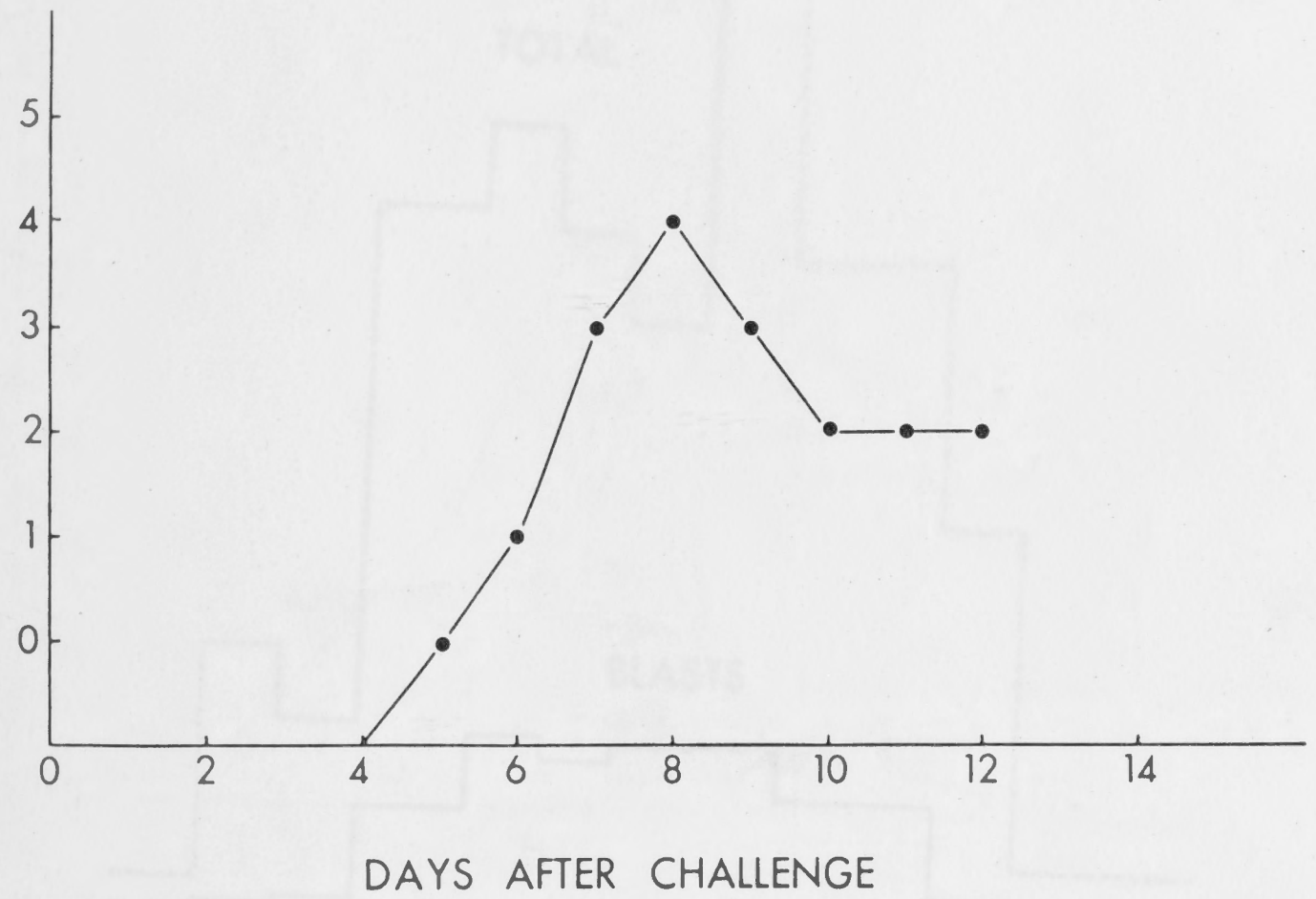


Figure 6.4 The binding antibody titre of the efferent lymph from the popliteal lymph node following challenge with autochthonous tumour cells in the presence of the primary tumour.

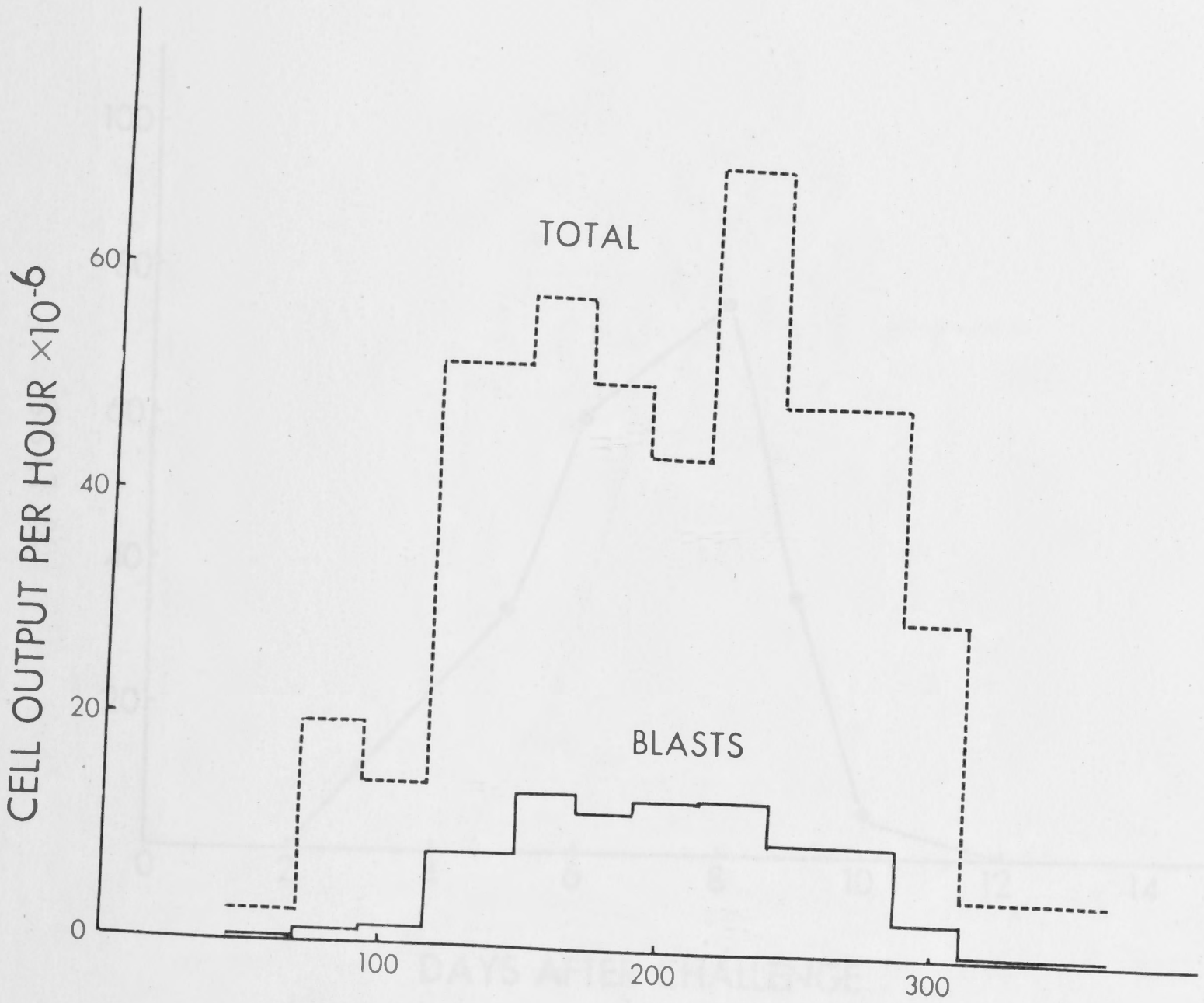


Figure 6.5 The cellular events occurring in the efferent lymph of the popliteal lymph node of sheep F35 following challenge with autochthonous tumour cells 3 weeks after removal of the primary tumour.

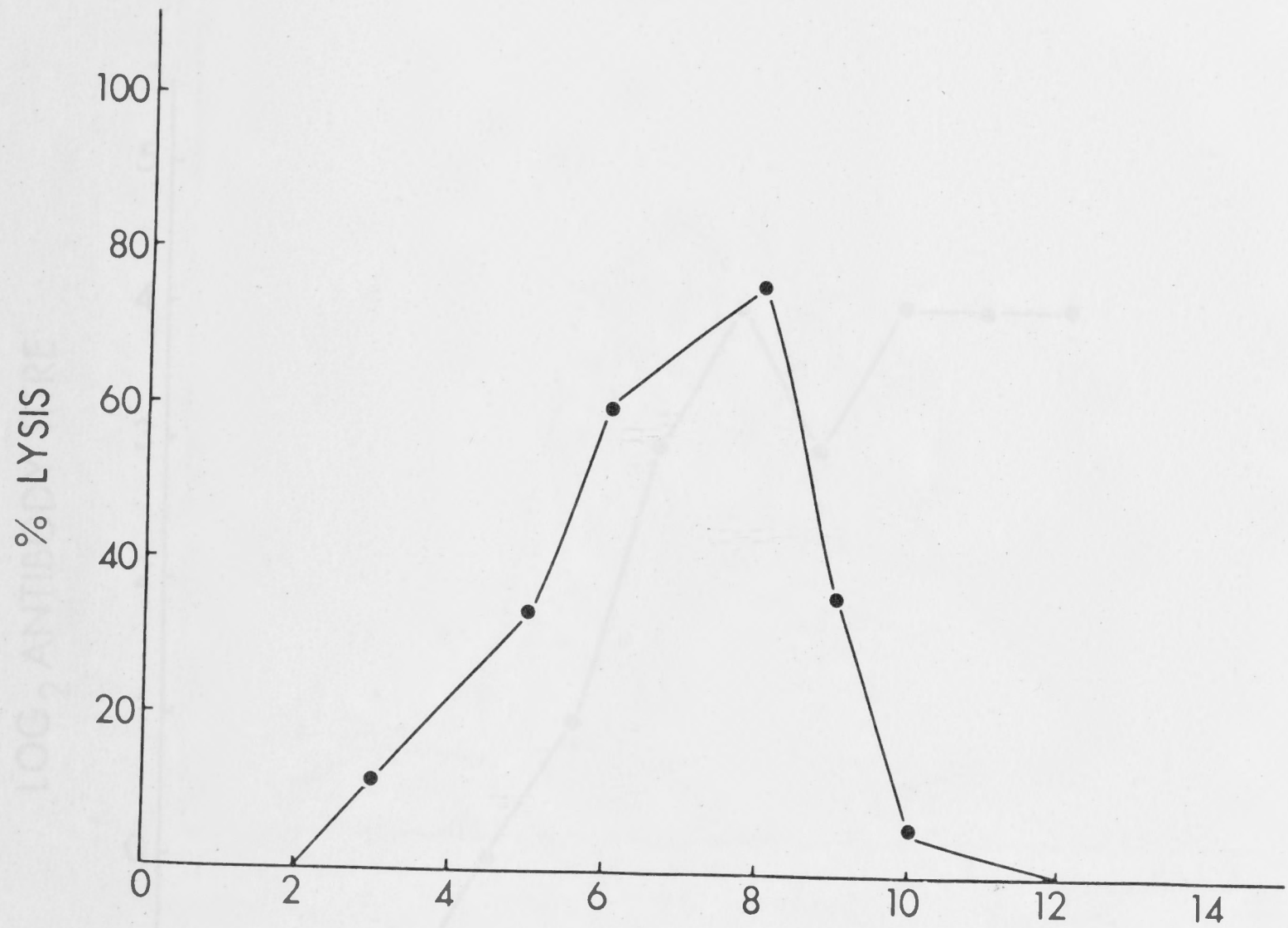


Figure 6.6 Cell-mediated cytotoxicity detected in the efferent lymph from the popliteal node following challenge with autochthonous tumour cells 3 weeks after removal of the primary tumour. Effector/target cell ratio, 200:1.

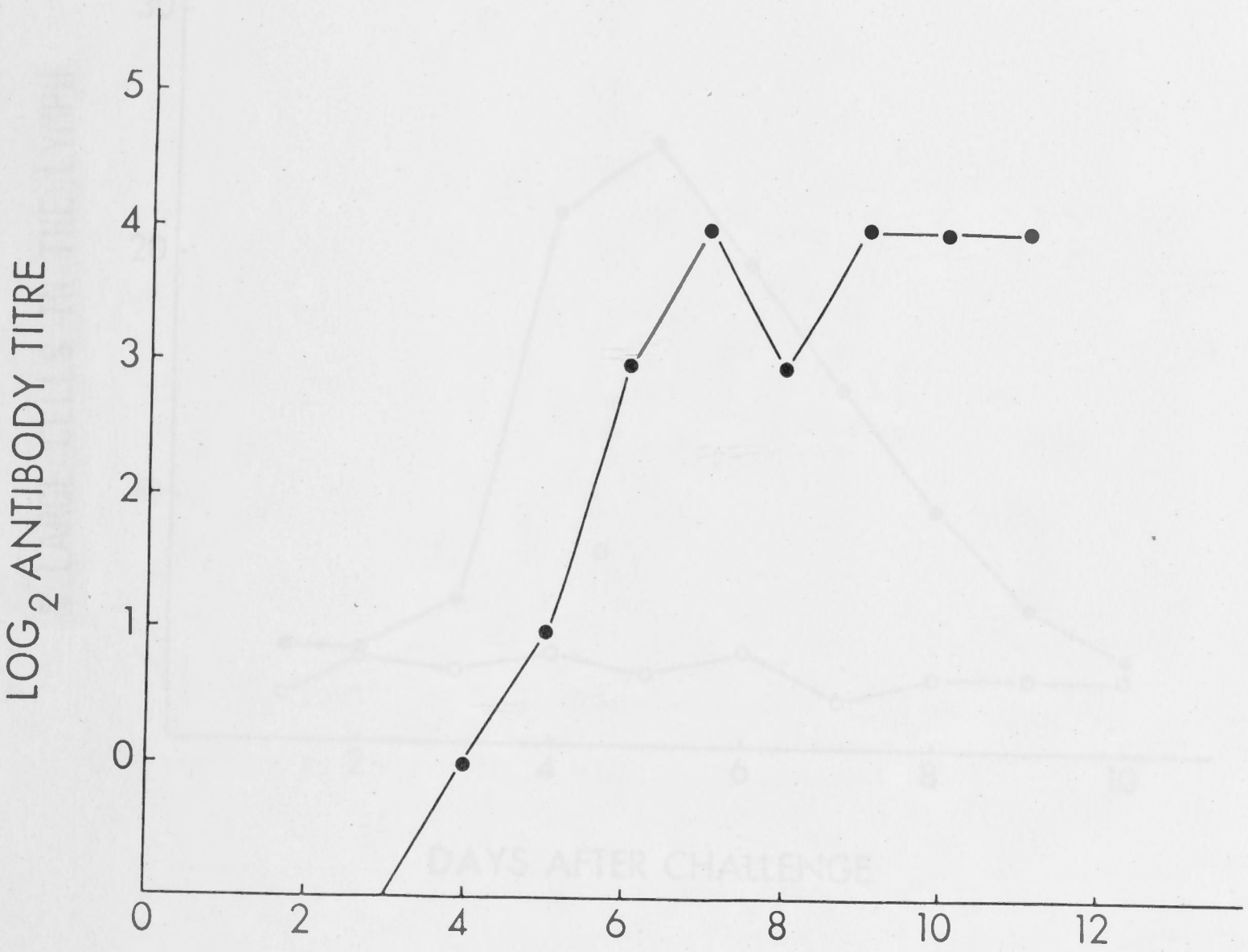


Figure 6.7 The cytotoxic antibody titre in the efferent lymph from the popliteal node following challenge with autochthonous tumour cells 3 weeks after removal of the primary tumour.

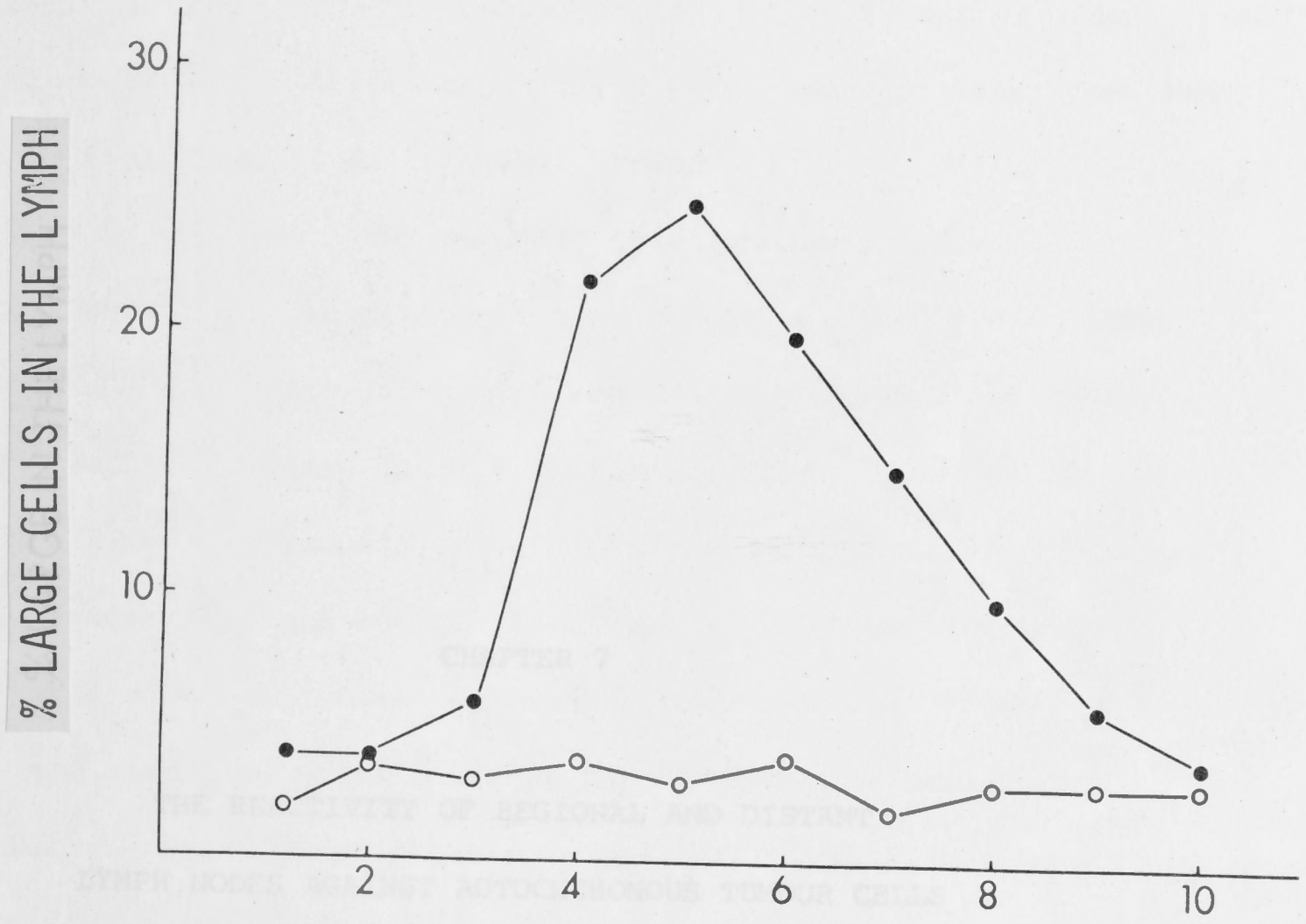


Figure 6.8 The percentage of blast cells in the efferent lymph from the popliteal node following challenge with equal numbers of autochthonous tumour cells (●—●) and epithelial cells (O—O).

CHAPTER 7

THE REACTIVITY OF REGIONAL AND DISTANT LYMPH NODES AGAINST AUTOCHTHONOUS TUMOUR CELLS

7.1 INTRODUCTION

Historically, the interpretation of the role played by the regional lymph node in determining the life-history of a developing cancer has been influenced firstly by the concept that lymph nodes act as mechanical filters that arrest any tumour cells carried to them in the lymph, and secondly that lymph nodes have a role in the immunologic rejection of tumours. Fisher and Fisher (1966, 1967a, 1967b) have shown that regional lymph nodes are not by any means effective filters of tumour cells. Thus, tumour cells injected intravenously traversed the blood capillary endothelium of lymph nodes and entered the lymph as do normal lymphocytes. It has also been shown that tumour cells are able to enter the blood stream by migrating from the lymphatic vessels directly through the walls of the blood capillaries within the substance of lymph nodes. This means that at least some cancer cells which have been shed from the primary tumour are able to undergo a blood-lymphatic circulation in both directions. Much of this cell traffic occurs within the substance of lymph nodes in a similar way to the normal traffic of lymphocytes. Zeidman and Buss (1954) however, demonstrated that lymph nodes of normal animals can be effective barriers to the spread of some tumours. They injected allogeneic Brown-Pearce or V2 carcinoma cell lines into the afferent popliteal lymphatics of rabbits and removed the lymph nodes 1 to 42 days after injection. Of a total of 30 animals injected, only 2 developed secondary tumours beyond the regional lymph node.

It is unquestionable that the regional lymph node is immunologically involved in the host's response to tumours. The

evidence for this comes from histological data (Alexander *et al.*, 1969; Flannery *et al.*, 1975; Berlinger *et al.*, 1976); the fact that it is possible to transfer immunity with lymph node cells (Delmore and Alexander 1964), and from the demonstrable interactions between tumour cells and regional lymph node cells (Fisher *et al.*, 1974).

The results of some clinical observations (Crile 1965; 1968; 1969) and experimental data (Fisher and Fisher 1971; 1972) on the role of regional lymph nodes in tumour immunity, have led to the proposal that it may be important to retain those regional lymph nodes which do not contain metastatic tumour so as to maintain a degree of systemic immunity against further growth of cancer. Although this concept has a significance for the clinical treatment of cancer and for tumour research, the evidence so far available for its support is inconclusive and conflicting. Fisher *et al.*, 1972, 1974) have suggested that the regional lymph nodes are important in the initiation and maintenance of tumour immunity. More specifically, they reported that immune cells from regional lymph nodes were more toxic for tumour cells *in vitro*, than were immune cells from other sources. This finding indicated that regional lymph node cells may be uniquely important in the host's defence against tumours. Accordingly Fisher *et al.*, (1974) suggested that the lack of histologic evidence of a growing tumour in the regional lymph node should not be interpreted as evidence that lymphatic metastasis has not occurred. Gardner and Rosen (1967); Bard *et al.*, (1969) and Hammond and Rolly (1970) have shown that regional lymphadenectomy has no effect on host immunity and they proposed that regional lymphadenectomy together with resection of the primary tumour should remain standard practice in

the treatment of cancer patients.

Most studies done to evaluate the role of the regional lymph node have been carried out on normal animals, which were often challenged with an overwhelming dose of tumour cells which usually produced death in a short period of time after inoculation. The pathogenesis of cancer as it occurs in man is not similar to this model since only the early events of the response in the regional lymph node to tumours can be studied (Sträuli 1970; Fidler 1974). As a result these experiments cannot be seen as counterparts of clinical studies which are more concerned with the later stages of the disease. Studies on the role of the regional lymph node in tumour immunity in large animals with spontaneous tumours are more comparable to the human situation.

The experiments reported in this Chapter were done to evaluate the reactivity of lymph nodes draining the site of progressively growing tumours and to compare this reactivity with that of lymph nodes distant from the site of tumour growth. Two different approaches were used to study this question. First, the *in vitro* anti-tumour reactivity of autochthonous lymphocytes obtained from the lymph node regional to tumour growth was investigated and compared with that of lymphocytes from a distant lymph node and with leucocytes from peripheral blood. Secondly, the responses occurring in the efferent lymph of both regional and distant lymph nodes were studied following challenge with autochthonous tumour cells.

7.2 RESULTS

7.2.1 *The Reactivity of the Regional Lymph Node, a Distant Lymph Node and Peripheral Blood Leucocytes Against Autochthonous Tumour Cells In Vitro.*

The regional cervical lymph node (RLN) and a distant popliteal lymph node (DLN) were surgically removed from 5 sheep which had tumours on their ears or their noses. The clincal data of the sheep studied are presented in Table 7.1. Lymphocytes isolated from these lymph nodes as well as peripheral blood leucocytes were tested for their cytotoxic reactivity *in vitro* against autochthonous tumour cells. Table 7.2 shows that no cytotoxic reactivity was apparent against autochthonous tumour cells in any of the animals, regardless of the source of lymphocytes.

7.2.2 *The Responses of the Regional Lymph Node and a Distant Lymph Node to Challenge with Autochthonous Tumour Cells*

In the experiments described here, the *in vivo* reactivity of tumour-bearing sheep to challenge with autochthonous tumour cells in the regional lymph node and in a distant lymph node was compared. The experimental protocol was similar to that used for the experiments described in Chapter 5. The afferent and efferent lymphatics of the cervical lymph node (RLN) and the popliteal lymph node (DLN) were cannulated and equal numbers (10^7) of tumour cells were infused into both nodes simultaneously, and the changes in the efferent lymph were monitored (Fig. 7.1).

(a) Challenge of distant lymph nodes

Four tumour-bearing sheep were challenged with autochthonous tumour cells into an afferent popliteal lymphatic. The clinical data of the sheep studied are summarized in Table 7.3. The response

Table 7.1 *The clinical data of the tumour bearing sheep studied in 7.2.1*

Sheep number	Sex	Age (years)	Tumour site	Tumour size (cm)	RLN	DLN
F31	female	7	ear	8x8	cervical	popliteal
F34	"	6	"	16x9	"	"
F39	"	7	"	15x12	"	"
F41	"	>7	nose	6x3	"	"
F47	"	>7	"	6x4	"	"

Table 7.2 *The reactivity in vitro of regional lymph node, distant lymph node and peripheral blood lymphocytes to autochthonous tumour cells*

Sheep number	% Cytotoxicity of lymphocytes from various sources		
	+ RLN	* DLN	† PBL
F31	-	-	1.2
F34	1.9	-	2.1
F39	-	1.3	1.1
F41	1.5	-	-
F47	-	-	-

\+ Regional lymph node

* Distant lymph node

† peripheral blood leucocytes

\- not detected

in this distant lymph node was similar to that observed in sheep challenged with autochthonous tumour cells. (See Chapter 6 Section 6.2.2).

Significant stimulation of the lymph nodes was obtained in all sheep studied and led to a substantial increase in the percentage of blast cells in the lymph. The changes in the efferent lymph consisted of an increase in the flow rate as well as an increase in the total and large cell output. There were changes detected 3 - 4 days following challenge and the response reached a peak on day 5. The total cell output and blast cell output remained at a high level up to day 12 after which the response declined. At the peak of the total cell output, the blast cells constituted 27% of the total cells in the lymph (Fig. 7.2). Differences in the responses were observed among the sheep tested, but while the timing and/or the magnitude of the response varied, the general pattern of the reaction was consistent in each individual sheep.

(b) Challenge of regional lymph nodes

The effect of autochthonous tumour cells on the lymph node regional to the developing tumour was investigated in tumour-bearing sheep which had been challenged with autochthonous tumour cells introduced into a distant lymph node. The lymph coming from the node regional to a growing tumour contained a high percentage of blast cells (10% - 14%). This may have been due to continuous stimulation of the lymph node by the inevitable bacterial contamination that occurs with a natural tumour and/or stimulation of the node by the tumour antigens or other products.

The results of the experiments in which the regional node was challenged with autochthonous tumour are presented in Fig. 7.3.

Before challenge, the total cell output was approximately 60×10^6 cells per hr while the large cell output was approximately 9×10^6 cells per hr. Following challenge the total cell output per hr decreased by 30% during the first 24 hr and then continued to decrease steadily throughout the experimental period (12-15 days). The large cell output declined in parallel with the total cell output and as a consequence the percentage of the large cells in the efferent lymph remained constant. It would appear from the results that, if an increase in the blast cell response is indicative of an immune response then these nodes were quite anergic, although the initial decline in the total cell output was similar to the "shut down" phenomenon described by Hall and Morris (1965a) in relation to antigenic recognition.

7.2.3 *Cytotoxic Activity in the Efferent Lymph of the Regional and Distant Lymph Nodes Following Challenge with Autochthonous Tumour Cells*

In all the sheep studied, efferent lymphocytes from both the RLN and DLN were tested for their cytotoxic properties against autochthonous tumour cells before (day 0) and at various intervals following challenge. No cellular cytotoxicity was observed in any of the cell populations from the sheep tested from either lymph node, using a variety of effector to target cell ratios in the cytotoxic assay, and periods of incubation up to 24 hr (Table 7.4).

Measurements of complement-dependent cytotoxic antibodies in the efferent lymph from both nodes were also done and no cytotoxic antibodies were detected at any time. This was consistent for all sheep studied.

Table 7.3 *The clinical data of the tumour-bearing sheep studied in 7.2.2*

Sheep number	Age (years)	Sex	Tumour site	Tumour size (cm)	Method of Challenge
F51	>7	Female	Ear	17x18	Cells via afferent lymphatic
F43	>6	Female	Nose	4x3	"
F59	>7	Female	Nose	7x10	"
F61	7	Female	Nose	6x3	"

Table 7.4 *Tests for cell-mediated cytotoxicity in the efferent lymph from the regional and distant lymph node following challenge with autochthonous tumour cells*

Days after challenge	Regional lymph node		Distant lymph node	
	% large cells	* % lysis	% large cells	* % lysis
0	14	1.0	2	1.9
2	13	-	4	-
4	13	2.5	13	2.0
6	16	-	18	2.6
8	13	3.0	25	1.5
10	10	-	21	3.0
12	13	2.3	10	-
14	10	1.1	8	1.3

* 24 hour assay at effector/target cell ratio, 200:1

\- not detected

7.2.4 The Specificity of the Response in the Regional Lymph Node

In order to determine whether the declining responsiveness in the regional lymph node was due specifically to the tumours, the distant lymph node (popliteal) of a tumour-bearing sheep was chronically stimulated by the subcutaneous injection of complete Freund's adjuvant into the lower part of the hind leg. Two months later, an afferent lymphatic of the popliteal node was cannulated and autochthonous tumour cells infused into the node as described previously. The response obtained was identical to that observed in the regional lymph node following challenge with tumour cells in that a decrease in the total and large cell output was observed. This result indicated that the reduced reactivity observed in the regional lymph node was a general phenomenon and that a distant node was also unable to respond normally to antigenic challenge (Fig. 7.4).

7.3 DISCUSSION

The purpose of the present studies was to examine the anti-tumour reactivity of a lymph node draining the site of a growing tumour and compare this reactivity with that of a lymph node remote in the body from the tumour. The results demonstrated that lymphocytes taken from the draining lymph node had no demonstrable cytotoxicity against the tumour cells; furthermore, there was no cytotoxic reactivity in peripheral blood leucocytes or in lymphocytes taken from a distant lymph node.

The reactivity of a distant lymph node challenged with autochthonous tumour cells suggested that while there were changes in the cell population in the efferent lymph which were indicative

of an immune response these changes were not associated with the production of any detectable anti-tumour cytotoxic mechanisms *in vitro*. Moreover the results obtained from the *in vivo* experiments demonstrated quite clearly that the regional lymph node did not respond to the tumour cell challenge by way of a blast cell response. The significance of the decline in the total cell output from the node following challenge with autochthonous tumour cells was ambiguous since a similar decrease in the cell output was observed in a node chronically stimulated by an unrelated antigen. These results suggested that the lymph nodes of the tumour-bearing sheep were generally debilitated and unable to produce normal immune responses.

This anergy displayed by the regional lymph nodes in tumour-bearing hosts has been described by other investigators. Clinical studies of cancer patients have revealed cases in which the blood leucocytes were cytotoxic for the patient's tumour cells while the regional lymph node cells were unreactive (Nairn *et al.*, 1971; 1972; Vänky and Stjernswärd 1971; Nind *et al.*, 1973). Studies from experimental animal models have revealed some details about the kinetic aspects of the development of regional lymph node unresponsiveness to tumour growth. Flannery *et al.*, (1973a, 1973b) investigated the immunological changes occurring in the regional lymph node draining the site of a growing squamous cell carcinoma in Wistar rats. They found that cytotoxic activity against the tumour was present in cells from the regional lymph node 2 weeks after the inoculation of tumour cells. Cytotoxic activity was also detectable in cells from intermediate and distant lymph nodes and in the spleen, but the appearance of this activity was out of phase with that observed in the regional lymph node.

The intensity of the cytotoxic reactions declined as the tumour grew so that eventually the lymphocytes from the regional lymph node became totally unresponsive while significant levels of cytotoxicity persisted in other lymphoid tissues. Flannery *et al.*, (1973a) suggested that such lymphoid anergy may be a factor in allowing the tumour to spread into the regional lymph node.

In another study with a chemically induced hepatoma (D192A) in rats , Jones *et al.*, (1978) reported that anergy of the regional lymph node, as demonstrated by cytotoxicity testing, developed 3 weeks following the implantation of the tumour. They found that anergy in the regional lymph node correlated with the finding that the paracortical (T-cell dependent) responses also declined towards the end of the 3rd week after implantation of the tumour. The B-cell dependent cortical areas of the cortex showed active lymphoid follicles two weeks after tumour transplantation and plasma cells appeared in the medulla. These reactions continued until the experiment was terminated 4 weeks after tumour implantation. Similarly, Alexander *et al.*, (1969) have shown that the anergy which develops in the regional lymph node is associated with changes in the proportions of blast cells and plasma cells in the regional lymph node. Thus it seems from these studies that the lymphoid anergy of a tumour-bearing animal is dependent on the duration and extent of the tumour growth. Moreover the anergy seems to be associated with a shift in the pattern of response in the node from a T-cell-dependent reaction to one which involves the B-cell-dependent response of the node.

The mechanism responsible for the development of the anergic state in the lymph nodes is not known. However, as described in Chapter 6, it is possible that tumour antigens or tumour antigen-

antibody complexes could be responsible for the creation of a non-reactive state in the lymphoid tissues possibly by binding to effector cells and causing their desensitization (Jones *et al.*, 1978), by preventing the differentiation of lymphoid cells into killer cells (Flannery *et al.*, 1975), or by immobilizing effector cells in the node and preventing them from being released into the circulation (Alexander *et al.*, 1969). Alternatively, tumours might cause anergy in the regional lymph node by flooding the node with suppressive factors which result in impairment of cell-mediated immunity either directly or indirectly through the activation of suppressor cells. Most of these possible explanations, however, are conjectural and are not supported by any experimental evidence.

In most of the experimental systems in which the phenomenon of lymph node anergy has been studied, the non-reactive state was specific for the tumour and its antigens, and the reactivity of the regional lymph node to other antigens remained normal. Moreover, the lymphoid anergy was geographically restricted to the regional lymph node (Alexander *et al.*, 1969; Flannery *et al.*, 1973a, 1973b). Lymphocytes taken from other sources were still reactive against the tumour. The situation was quite different with the naturally occurring skin cancer in sheep. Jun *et al.*, (1979) reported that the reactivity of peripheral blood leucocytes against tumour antigens and other mitogens was decreased in tumour-bearing sheep, suggesting that there was a degree of systemic lymphoid anergy. The diminished reactivity also had both specific and non-specific components to it. Since it has been shown by several investigators that the development of regional lymphoid anergy is associated with a shifting pattern of reactivity from cellular to humoral responsiveness, (Flannery *et al.*, 1973a, 1973b, 1975; Jones *et al.*,

1978), it is possible that responses observed in distant lymph nodes (antibody response) are a reflection of a changing reactive pattern that has already occurred in the regional lymph node. The difference in the pattern of response between a regional and a distant node may also be due to differences in the extent to which each node has been subjected to stimulation.

The lack of reactivity of lymphoid cells from the regional and distant lymph nodes against the tumour may have some significance for the secondary growth of the tumour cells which occurred in them following the inoculation of tumour cells. Metastatic tumours were detected in 2 out of 9 of the distant nodes inoculated with tumour cells 15-20 days previously (Fig. 7.5), and 1 out of 4 of the regional nodes. It should not be construed that metastastic growth was absent in nodes in which no tumours were found as no attempt was made to section the nodes serially.

Thus it is possible that the regional lymph node may play an important role in tumour rejection at an early stage in the development of the primary tumour. It has been shown experimentally that the barrier function of the regional lymph node for tumour cells is affected by the number of tumour cells and the period over which they are being disseminated to the node (Sträuli 1970).

It seems likely that early in the life-history of a tumour when it is small and the lymph nodes are not affected, it may be detrimental to the development of the host's immunity to the tumour to remove the regional nodes. This in itself may increase the likelihood of metastasis occurring (Crile 1969). Late in the course of the disease when the nodes become anergic or when they contain metastatic tumour they may no longer be important in the generation of systemic immunity and their removal may be of less

consequence. Many human cancers are diagnosed after the disease has been developing for some time and in many cases the regional lymph node may already have become anergic. The present findings suggest that there is some therapeutic justification for removing the regional lymph nodes in the later stages of clinical carcinomatosis.

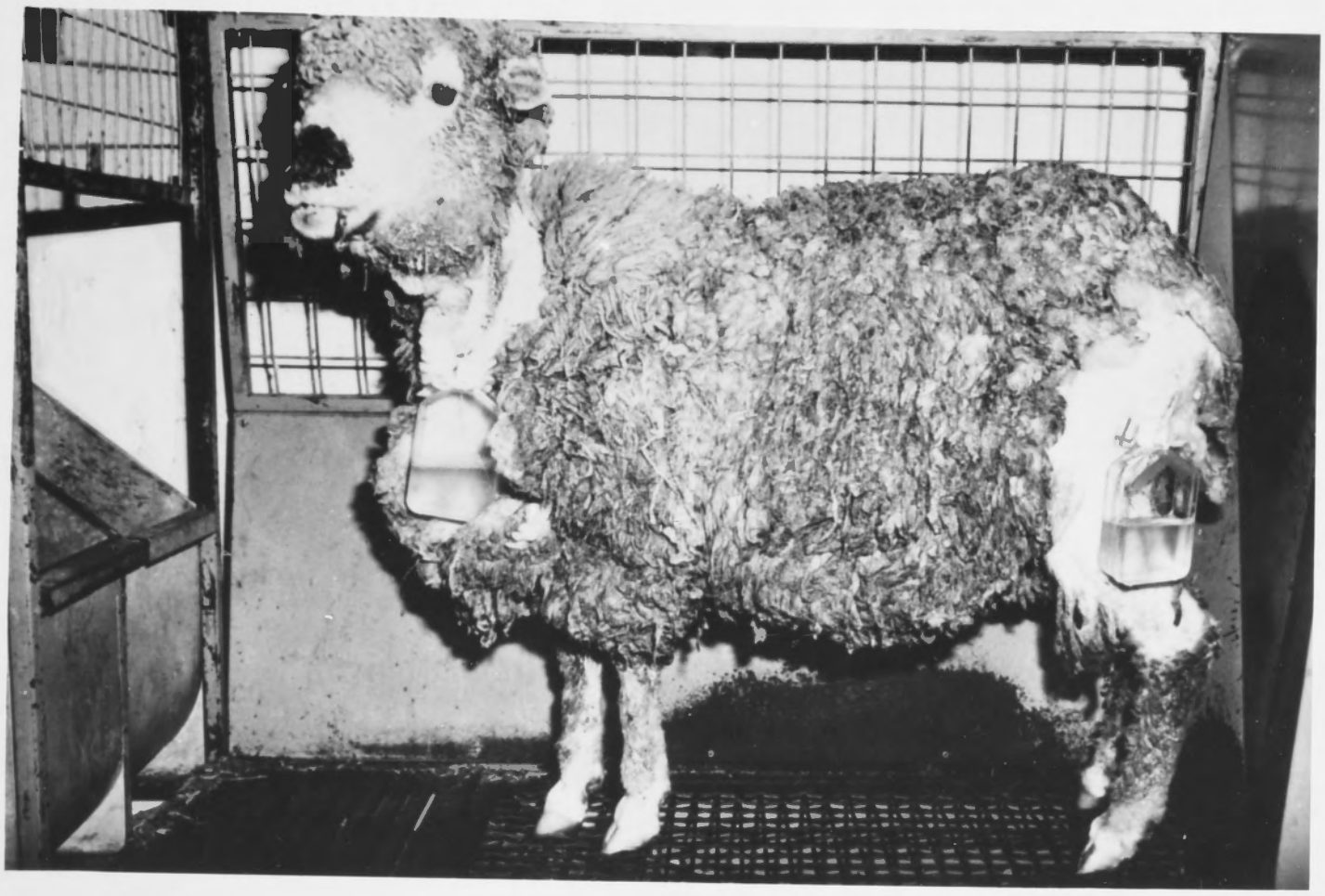


Figure 7.1 Experimental arrangements for studying *in vivo* reactions to autochthonous tumour cells in the regional and distant lymph nodes. The afferent and efferent lymphatics of the regional (cervical) and the distant (popliteal) lymph nodes of tumour-bearing sheep were cannulated and the sheep were challenged with autochthonous tumour cells introduced via the afferent lymphatic. The subsequent responses in the cervical and popliteal lymph nodes were followed by monitoring changes in the efferent lymph.

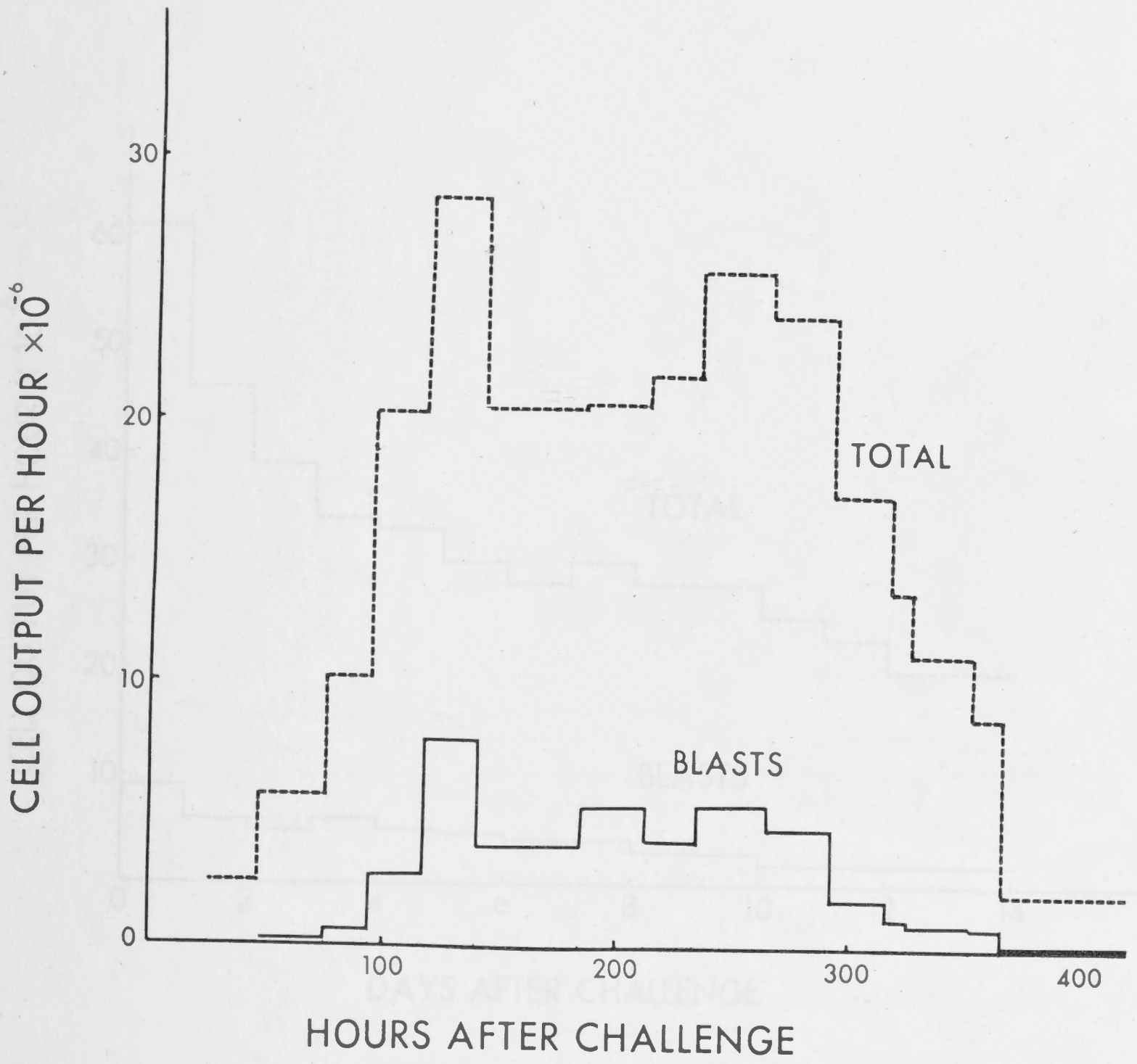


Figure 7.2 The cellular events occurring in the efferent lymph of a distant lymph node (popliteal) following challenge with autochthonous tumour cells.

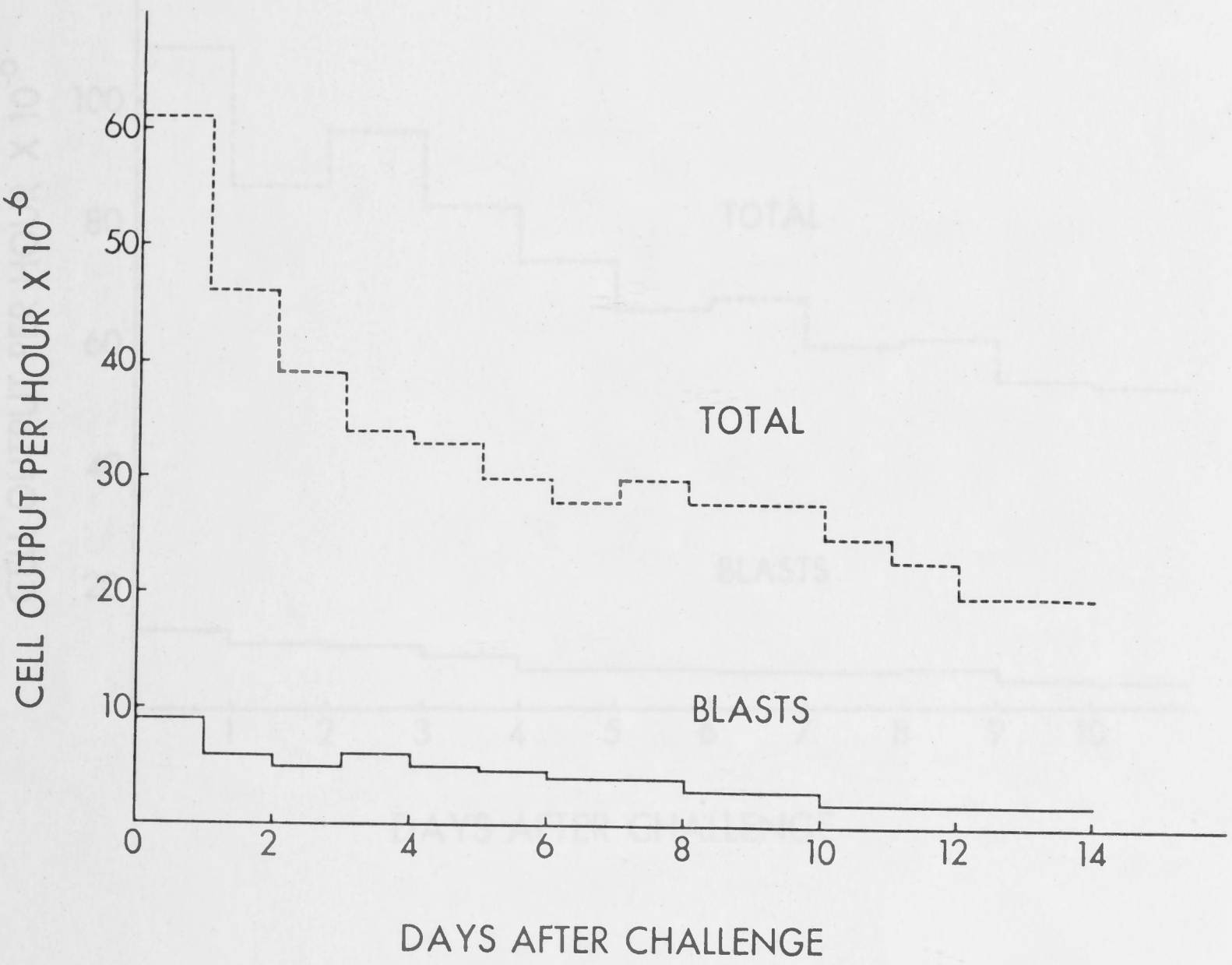


Figure 7.3 The cellular events occurring in the efferent lymph of the regional lymph node (cervical) following challenge with autochthonous tumour cells.

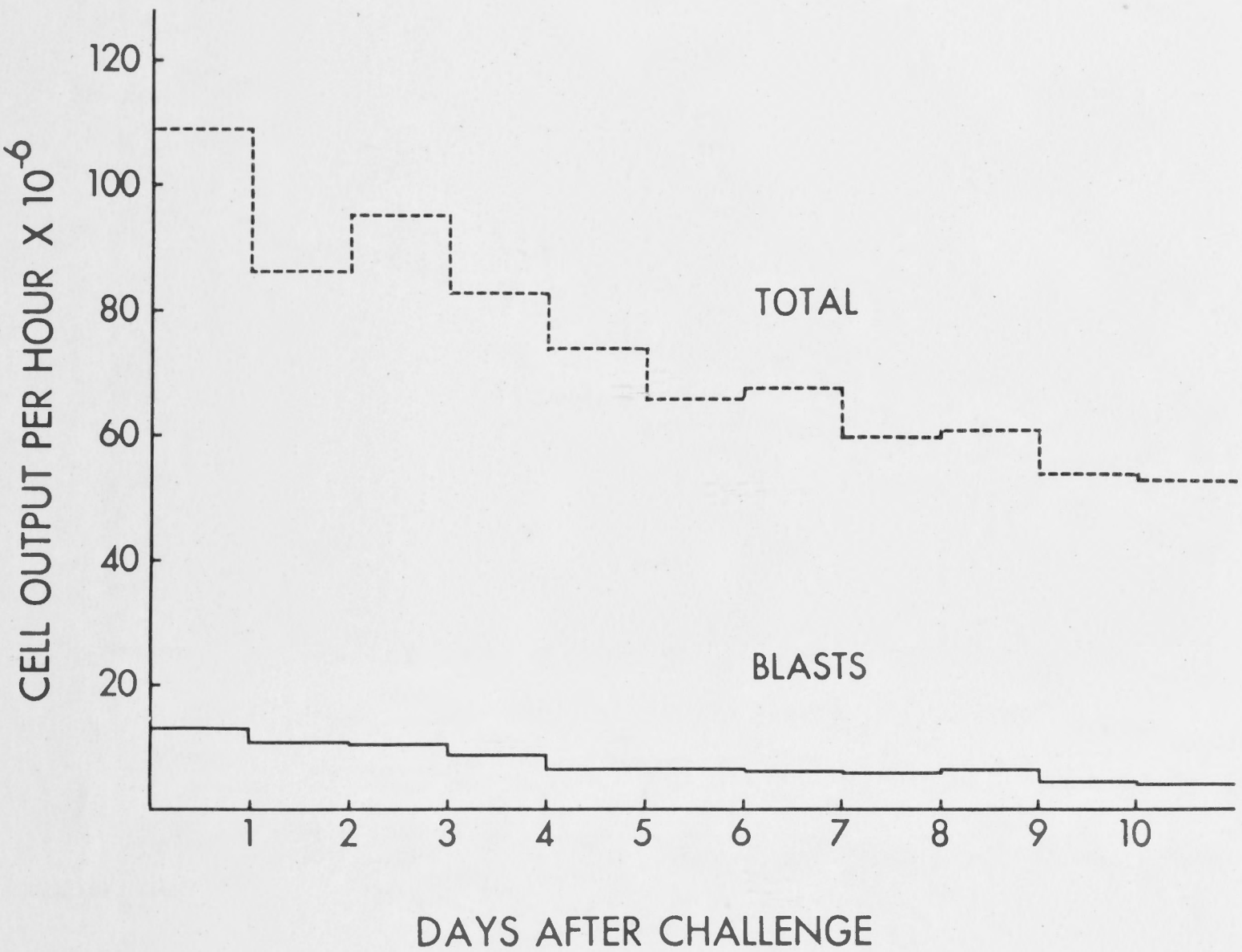
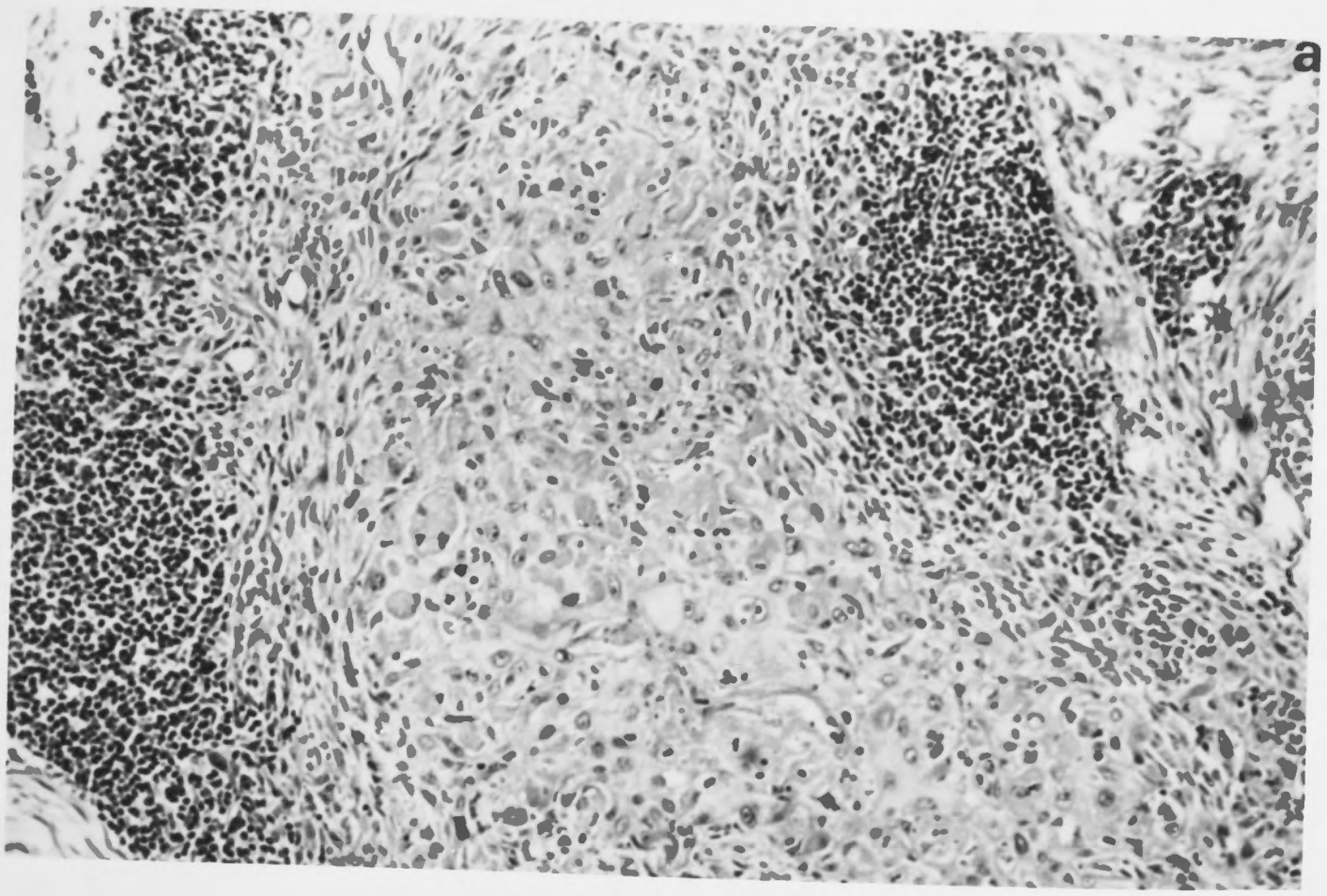


Figure 7.4 The cellular events occurring in the efferent lymph of a distant lymph node (popliteal) chronically stimulated with CFA and challenged with autochthonous tumour cells.

Figure 7.5a A secondary tumour growth in a distant lymph node (popliteal) challenged with autochthonous tumour cells. The node was sampled 20 days following the introduction of tumour cells into it (magnification X125).

Figure 7.5b The histological appearance of the primary tumour from which the inoculated cells were cultivated (magnification X125).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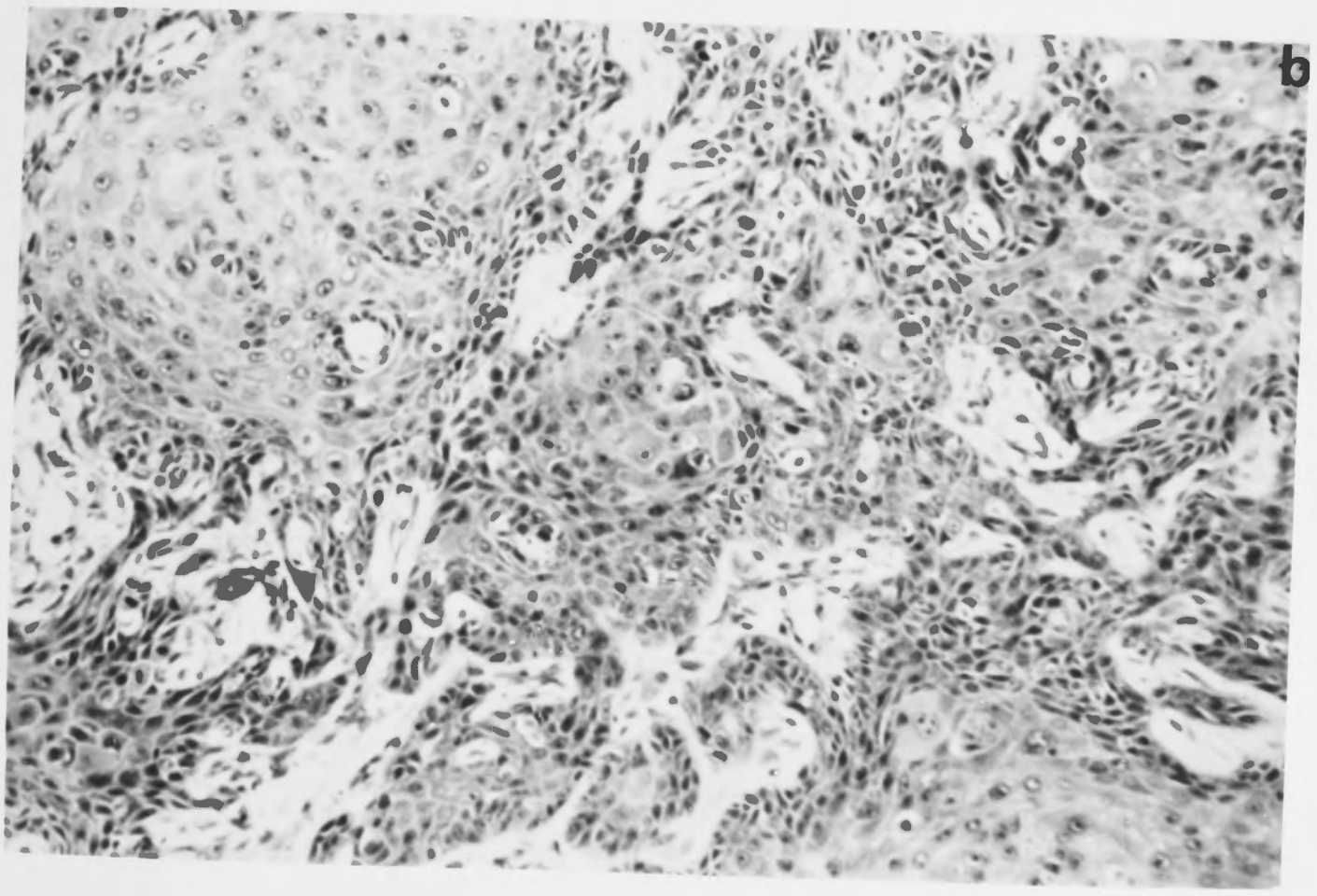
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CHAPTER 8

GENERAL CONCLUSIONS

The discovery that neoplastic cells express specific antigens which could be recognized by the host's immune system has opened up a new era in cancer research. This discovery provided a theoretical basis for the use of immunotherapy in cancer treatment and offered the advantage of specificity which other conventional forms of therapy such as surgery, radiotherapy and chemotherapy, do not have. If a specific destructive immune response could be generated in a cancer patient, it may be possible to eliminate the neoplastic cells with very little damage to normal tissues. In addition to specificity in the destruction of the tumour, the resultant immunological memory may contribute to a continuing immune surveillance against any recurrence of the tumour.

Subsequent to the discovery of the presence of tumour specific antigens on neoplastic cells, a variety of tumours were induced experimentally in laboratory animals and investigations were carried out to clarify and understand the interactions that occur between the tumour cells and the immune system of the host. During the course of these studies a number of anti-tumour effector cells and mechanisms were defined. However, it soon became obvious that most models of experimentally induced tumours were not analogous to naturally occurring human cancers. These experimental models were dependent on the use of syngeneic animals for the supply of both tumour tissue and sensitized cells for adoptive transfer experiments. The responses seen in healthy, syngeneic recipients, challenged with a sudden overwhelming dose of tumour have no counterpart in the responses mounted by animals in which long-standing tumours are growing. Accordingly, there is a need for the reappraisal of results obtained from such models and for the study of responses to tumours which have a much

greater similarity to human cancer (Alexander 1976; Bartlett *et al.*, 1976; Hewitt 1978).

A problem which emerged during the studies of the host-tumour interactions, related to the interpretation of *in vitro* experimental results in tumour immunology. It is generally accepted that some of the interactions taking place between the host and the tumour can be interpreted from the results of *in vitro* confrontations between lymphocytes and tumour cells. However, results from various laboratories have indicated that this assumption may not be true (Ilfeld *et al.*, 1973; Baldwin and Embleton 1974), and as a consequence there is a need for doing both *in vitro* and *in vivo* studies in order to understand fully the nature of host-tumour interactions.

Naturally-occurring squamous cell carcinomata in sheep offer a model for studying host-tumour interactions in a situation which bears a close resemblance to a common type of human cancer. The tumour appears to have an aetiology which has been attributed to ultraviolet irradiation, the main causative factor in the induction of skin cancer in humans. Sheep represent an outbred population of animals with a relatively long life span and a large body size. In addition the tumour-bearing sheep are frequently debilitated, all of which are similar to some of the features that are associated with human cancer. Furthermore, sheep have the added advantage for *in vivo* studies of immune responsiveness, in that reactions in single lymph nodes can be continuously monitored by establishing chronic fistulae in the regional efferent lymphatic ducts.

The interaction between a tumour and the host's immune system can be studied from the standpoint of the properties of the tumour cells as well as the tumour cell interaction with the host.

Accordingly, initial studies were done to examine some of the properties of the tumour cells themselves by investigating the tumour cell-allogeneic lymphocyte interaction *in vitro* and *in vivo*. Results from the *in vitro* experiments revealed that none of the cell lines tested were able to stimulate a proliferative response in allogeneic lymphocytes. This inability to stimulate was not due to compatibility between the responding and stimulating cells or to lack of alloantigen expression by the tumour cells. In the majority of the cell lines tested, the failure to stimulate was probably due to an immunosuppressive effect of the tumour cells.

Investigations of the capacity of these particular cell lines to stimulate *in vivo* showed that they produced significant stimulation which was manifest by the appearance of both humoral and cellular cytotoxic mechanisms directed against the tumour. These results indicated that the suppression induced by tumour cells *in vitro* had no effect *in vivo*. These data again stressed the importance of interpreting the *in vitro* studies with the knowledge that they may not predict the behaviour of the tumour *in vivo*.

Although no suppressive activity of the tumour cells was observed in allogeneic sheep *in vivo*, this does not rule out the importance of such a suppressive mechanism playing a role in the tumour-bearing host. In animals debilitated by a long-standing tumour, the antigenic disparity between the tumour and the host may not be as strong as in an allogeneic healthy sheep. This may result in a shifting of the outcome of the response in favour of tumour growth which in turn may lead to immunosuppression.

In light of the differences in the characteristics of the various cell lines and with the knowledge of the limitations

these differences may impose on the immune response of a tumour-bearing host, the host's ability to respond to its own tumour was investigated. The results indicated that no cytotoxic anti-tumour reactivities, either cellular or humoral were detected against the injected tumour cells. However, antibodies that could specifically bind to the tumour but were not cytotoxic were detected following challenge. The lack of reactivity was found to reflect a systemic depression of the immune response against the tumour, since no anti-tumour cytotoxic reactivities were detected in the regional lymph node, in the distant lymph node or in the blood. This anergy of the lymphoid tissue may account for the artificial metastasis of these tumours seen in both the regional and distant lymph nodes following challenge with tumour cells.

Removal of the primary tumour did not result in any restoration of the reactivity of lymphoid cells when tested three weeks after the tumour was resected. However, subsequent sensitization of the host 3 weeks after removal of the tumour was associated with the appearance of both cellular and humoral cytotoxic responses directed specifically against the tumour.

The results of these experiments indicated quite clearly that these naturally occurring tumours are antigenic and, under proper circumstances, they are capable of stimulating an immune response in the host. The demonstrable immunogenicity of these tumours raises again the paradox as to how these recognizably antigenic tumours escape immune destruction.

A wide variety of speculative theories have been put forward to explain how antigenic tumours evade the host's immune defence. Some of these theories are supported by experimental

evidence, but the importance or even the relevance of any of these mechanisms to the life history of a tumour is entirely conjectural. One obvious characteristic of the tumour cells which could enable them to escape immune destruction by the host is their immunosuppressive effect on the host lymphoid apparatus. Studies with squamous cell carcinomata in sheep by another group of investigators (Jun 1979; Jun *et al.*, 1978) have shown that the injection of tumour extracts into tumour-bearing sheep caused an increase in the growth of the tumour (enhancement) and its metastatic spread. Comparable enhancement was also associated with the inoculation of tumour-bearing sheep with foetal lamb skin. These investigators suggested that the enhancement of tumour growth was a result of suppression of the immune system of tumour-bearing sheep by the tumour and by foetal tissue extracts. This led them to suggest that tumour growth was associated with the re-expression of some foetal tissue product.

The basic question of tumour biology that requires investigation concerns the role of the immune system of the host in explaining the clinical behaviour of tumour growth and in particular the metastatic potentiality of neoplastic cells. Research into this facet of tumour biology should be done with the knowledge that immunological reactions of the host and the antigenicity of the tumour cells are not the only facets which may affect the outcome of a cancer metastasis. A basic understanding of other aspects of the biology of tumour cells which enables them to metastasize is central to the problem of tumour growth in the host. For the host immune system to be able to play a role in controlling tumour spread two criteria must be operative. 1. the tumour cells must be antigenic, and 2. the host immune

system must be able to recognize and destroy these cells by immunological means. The variable antigenic properties of neoplastic cells makes the control of tumour spread by immunological means a limited and a difficult biological task. An understanding of the circumstances in which the immune system might contribute to the control of malignant processes would offer prospects for cancer therapy but there is no certainty in the proposition that immune reactions demonstrable *in vitro* will necessarily be operative *in vivo* in cancer control. Much more needs to be understood about the intricacies of host-tumour interactions *in vivo*, particularly in relation to the chronic evolutionary changes that must inevitably occur between an evolving tumour and an evolving reaction to it by the host. The naturally-occurring squamous cell carcinoma of the sheep provides a model in which these interactions can be studied.

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